UNIVERSITY OF TASMANIA

Department of Zoology

Final Report on

FIRTA/FIRDC Project No 87/92

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Final Report on FIRTA/FIRDC Project no 87/92 'Assessment of restriction enzyme analysis of mitochondrial DNA for the identification of stocks of commercially important marine species and for the detection of genetic markers for use in salmonid husbandry'

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CONTENTS

1. PROJECT TITLE

Assessment of restriction enzyme analysis of mitochondrial DNA for the identification of stocks of commercially important marine species and for the detection of genetic markers for use in salmonid husbandry

2. AFFILIATION

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4. PROJECT AIMS

Six objectives for this project were submitted in the Initial Application. These are given below.

1. To establish a system of collecting samples of marine fish from which pure, undegraded DNA can be extracted in the laboratory, from the geographic range of the species.

2. To apply restriction enzyme analysis to the mitochondrial DNA samples.

3. From the results of the restriction enzyme analysis, to calculate the amount of genome similarity between and within populations of the species to identify any patterns of similarity.

4. To relate information about the similarity of the genomes to known aspects of the population biology of the species in an attempt to describe the dynamics

of the genome in marine species.

5. To use restriction enzyme analysis of mtDNA to survey the amount and type of genetic diversity in rainbow trout and Atlantic salmon in Australia.

6. To determine if the mitochondrial DNA genome can be used as a genetic marker in selective breeding programs of cultured trout and salmon.

In the Initial Application it was proposed to work on four marine species for which it appeared that immediate management problems required information on the genetic structure of stocks. The fours species were jack mackerel, orange roughy, gemfish and blue grenadier.

During the first year (1987/88) some work was conducted towards achieving all six objectives. However, in 1988 the newly established Fishing Industry Research and Development Committee requested that the project be restricted to no more than two species of marine fish and that particular attention be given to the possible identification of genetic structuring of populations of orange roughy. Accordingly, all work on the blue grenadier and gemfish was immediately stopped. A preliminary study of the genetic profiles of four salmonid species in Tasmania was more advanced; therefore, the work relating to Objective 5 was continued for several months on a low priority basis until such time as a publishable amount of data was obtained. For the remainder of the project most attention was given to the orange roughy. Data on jack mackerel were also collected and analysed.

Work and results of each of the objectives are summarised below; fuller details are provided in the published papers given in the Appendices.

5. BACKGROUND

Mitochondrial DNA in animal cells is a small, covalently closed, circular molecule, 16 to 20 000 base pairs long. Animal mtDNA undergoes relatively

simple forms of evolutionary change and is clonally inherited through the female lineage; therefore the genome is uncomplicated by recombination (Avise *et al.* 1987, Birky *et al.* 1989). The relative rate of mtDNA evolution is 5 to 10 times faster than single-copy nuclear DNA, or 0.02 substitutions per base pair per million years (Brown *et al.* 1979). Restriction-enzyme analysis of mtDNA estimates the relative amount of nucleotide-sequence diversity in the mitochondrial genome and, by inference, the degree of genetic subdivision between animal groupings. The assumption is that once a barrier to gene flow has been established between individuals from the same population, different mutations in the mtDNA will arise and be perpetuated within each subpopulation.

It was anticipated that the greater resolving power of mtDNA analysis, as opposed to other molecular techniques such as allozyme electrophoresis, may provide a better means of identifying the stock boundaries of commercially important marine fish species.

Further background on the application of mitochondrial DNA analysis to the identification of genetic structuring of fish populations is given in a major review by Ovenden (1990) which is reprinted in Appendix B. This paper summarises the state of knowledge as at the commencement of this project; some results from this study are also incorporated in the paper. It was prepared as part of this project and formed the basis of a presentation to a workshop on marine stock assessment held by the Australian Marine Sciences Association in Sydney in 1988.

6. OBJECTIVE 1

To establish a system of collecting samples of marine fish from which pure, undegraded DNA can be extracted in the laboratory, from the geographic range of the species.

The preferred method for collecting samples for the analysis of DNA involves the harvesting of tissue within minutes of the death of the animal and immediately freezing this tissue quickly at very low temperatures. This usually involves using liquid nitrogen or having access to a very low temperature freezer (<-80° C). Although facilities for this method of collecting are available in many laboratories and on some research vessels, it is logistically difficult to obtain them in many locations and impossible to do so when commercial operators are used to provide samples.

We compared the DNA yield from different fish tissues which had been stabilized under various conditions. It was immediately apparent that the highest relative yield of clean DNA is obtained from mature oocytes in the ovary. However, such material is not always available and other tissues which provided good yields were sub-mature ovaries, testes, heart and liver.

A comparison of the effects of storage regimes on the quantitative yield of DNA from the same tissue type indicates that the greatest recovery was obtained from fresh tissue followed by samples immediately frozen in liquid nitrogen. However, acceptable yields were obtained from tissues frozen in a commercial blast freezer operating at <-25° C and tissues stored for up to 2 months in TEK (TRIS-EDTA-potassium) buffer at +4° C. These two latter techniques clearly provide means by which samples for genetic analysis can be obtained from commercial fishing boats.

7. OBJECTIVE 2

To apply restriction enzyme analysis to the mitochondrial DNA samples.

Each new species and tissue combination presents special problems for the successful analysis of mitochondrial DNA using restriction enzyme

techniques. We were keen to develop techniques for work on fish species which could be conducted in only moderately equipped and funded laboratories. For this reason, we eschewed the use of an ultracentrifuge for the purification of the DNA. Ultracentrifugation, using a caesium chloride gradient system, involves the use of a very expensive piece of equipment and, because of the length of time and cost of the procedure, mitigates against the treatment of the large numbers of samples often needed for studies of the genetics of populations. Instead, we modified the method of Chapman and Powers (1984) which uses a phenol-chloroform system to isolate mitochondrial DNA without the use of an ultracentrifuge. Our modifications are explained in Ovenden, White and Sanger (1988).

The phenol-chloroform technique is now used regularly to prepare DNA for treatment with restriction enzymes which recognize and cleave the genome at a sequence of six nucleotides (6-base enzymes). We were able to refine the technique to enable the routine purification of DNA to be examined using the more informative system involving the use of 4-base enzymes which cleave the DNA at sites comprising four nucleotides. In most other laboratories this system still depends upon access to an ultracentrifuge. Indeed, a referee of one paper emanating from this project (Smolenski, Ovenden and White 1993) initially challenged our findings on the stock structure of the orange roughy on the basis of his assumption that it was not possible to prepare DNA of high enough purity for 4-base analyses using the phenol-chloroform method. He relented only after we provided copies of autoradiographs of our electrophoretic gels in which the products of 4-base enzyme digestion had been separated.

This method of analysis was used successfully throughout this project. It was also been applied in another FIRDC-funded project (Project 88/41 - Pilot study of larval recruitment and genetic variation of southern rock lobster

populations), the results of which were published in Brasher, Ovenden, Booth and White (1992), Brasher, Ovenden and White (1992), Ovenden, Brasher and White (1992) and Ovenden and Brasher (1993).

8. OBJECTIVE 3

From the results of the restriction enzyme analysis, to calculate the amount of genome similarity between and within populations of the species to identify any patterns of similarity.

8a Orange roughy

Initially we conducted a preliminary study of the genetic profiles of orange roughy populations from off the west (n=23) and east (n=26) coasts of Tasmania. The variation between the two sites was not significantly significant. However, enough differences were detected between west and east Tasmania for us to speculate that if the pattern was confirmed by studies of larger samples then it would be safe to conclude that the gene flow between the orange roughy populations was restricted in some way. The results of this preliminary study are detailed in Ovenden, Smolenski and White (1989) which is given in Appendix A.

We then proceeded to a study based upon samples collected over most of the geographic range of orange roughy in the Southern Hemisphere. Two hundred and eighty-six range roughy samples were collected from seven general localities: the Great Australian Bight; South Australia (off southeastern Kangaroo Island; the west coast of Tasmania; the east coast of Tasmania; New South Wales; New Zealand and South Africa (see Fig 1 in Appendix E for map of sampling locations).

Mitochondrial DNA was extracted from developing ovary tissue and analysed with ten 6-base and three 4-base enzymes. Both forms of analysis revealed a low level of genetic diversity in this species. The 6-base enzyme study found

no evidence of reproductively isolated populations in southeastern Australian waters. However, an analysis of 107 fish with three 4-base enzymes identified at least partial genetic separation of the New South Wales sample from the South Australian and Tasmanian samples. This finding supports biological evidence for the presence of a distinct subpopulation in NSW waters.

The 4-base study also provided evidence of the presence of genetically distinct samples of orange roughy occurring in the same localities off southeastern Kangaroo Island from consecutive years. There was no indication that the samples of orange roughy collected from South Africa and New Zealand were genetically different from those obtained from southern Australian waters.

The results of this main study are detailed in Smolenski, Ovenden and White (1993) which is given in Appendix E.

8b Jack mackerel

A preliminary investigation of the genetic structure of jack mackerel in southeastern Australian waters was conducted using restriction enzyme analysis of mitochondrial DNA. Seventy five jack mackerel were collected from two localities: off Eden in New South Wales and off southeastern Tasmania. Fish were sampled twice at each locality.

The 75 jack mackerel samples were screened with 11 6-base enzymes and four 4-base enzymes. The level of genetic diversity in jack mackerel was low. The 6-base analysis found limited evidence of reproductive isolation between New South Wales samples. The analysis based on 4-base enzymes also suggested temporal differences in the mtDNA diversities of the two Tasmanian samples of jack mackerel.

The results of the jack mackerel study are detailed in Smolenski, Ovenden and White (1994) which is given in Appendix F.

9. OBJECTIVE 4

To relate information about the similarity of the genomes to known aspects of the population biology of the species in an attempt to describe the dynamics of the genome in marine species.

The orange roughy and jack mackerel have very different biologies. The former is a very long-lived species living in deep waters (Fenton, Short and Ritz 1991). The latter is a relatively short-lived pelagic species (Stevens and Hauslied 1982). It might be expected that the genetic dynamics of the two species would be different.

This study revealed a low level of genetic variation in the two species. However, evidence was also obtained to indicate that some reproductive isolation between both orange roughy and jack mackerel in New South Wales waters and those from more southerly locations

A possible limited dispersal capability of adult orange roughy may account for the apparent reproductive isolation of the New South Wales sample. There is evidence from observations in New Zealand that orange roughy may travel up to 200 km to reach a spawning area (Bell *et al.* 1992). It remains unclear as to how far orange roughy in Australian waters migrate in order to aggregate at a spawning or feeding ground. Circumstantial evidence of orange roughy migrations off the east coast of Tasmania has arisen from observed changes in the proportion of non-reproductive fish, due to the movement of mature individuals just prior to spawning (Bell *et al.* 1992). However, the pattern of migration is not well understood.

Although information on the larval biology of orange roughy remains incomplete, it appears that the greatest potential for dispersal in this species possible occurs during the zygote and, to a lesser extent, the larval phase. Pankhurst, McMillen and Tracey (1987) reported that fertilized eggs are pelagic and ascend to surface waters following spawning. During this time the zygotes can, potentially, be carried considerable distances by currents. However, more recently, T. Koslow (CSIRO Division of Fisheries, personal communication) has suggested that orange roughy eggs occur mainly at depths similar to that occupied by the adults (700 to 900 m) and are rarely found at shallower depths.

Reproductive isolation may be maintained between Tasmanian and New South Wales stocks as the East Australian current (EAC) diverts eastwards towards New Zealand at ~33° S. The remainder of the EAC continues to southward in a series of surface eddies towards Tasmania (Heath 1985). It is possible that the orange roughy larvae, if they are restricted to deeper waters as claimed by Koslow, may not be trapped in these eddies and are therefore not transported southward towards Tasmania. Furthermore, the northeasterly flow of the EAC in the Tasman Sea may contribute to the separation of New South Wales and New Zealand stocks of orange roughy due to the larvae being carried well north of New Zealand (Hamilton 1990). Brasher *et al.* (1992) suggested the same hypothesis to support preliminary evidence for genetic separation of stocks of the palinurid lobster, *Jasus verreauxi*, between New South Wales and New Zealand.

The presence of possibly discrete stocks of orange roughy in southeastern Australia has major implications for the management of this resource. Factors such as the long-lived nature of orange roughy (Fenton, Short and Ritz 1991), intermittent spawning (Bell *et al.* 1992) and the possible low vagility of adult fish, make this species susceptible to over-fishing.

Although the level of genetic diversity in jack mackerel samples was low, there was some evidence of temporal differentiation. Richardson (1982) conducted a study of the population genetics of this species using allozyme electrophoresis and concluded that there was some evidence of genetic differentiation between schools of fish. Sharp (1978) had suggested a similar phenomenon for tuna (*Thunnus* spp). Our evidence remains inconclusive on this topic.

The low levels of genetic diversity in jack mackerel may be considered to be surprising given the apparently very large effective population size of the species. However, it is possible that the loss of a large proportion of larval jack mackerel due to a combination of starvation, poor or no advection to inshore waters and predation may adversely affect subsequent recruitment, resulting in the possible extinction of some mtDNA lineages. Such losses would have the effect of of reducing the real effective population size of the spawning aggregations, thereby contributing to a lower overall mtDNA diversity.

Further, the ability of modern purse-seine vessels to remove almost entire schools during a single netting operation may also have a major long-term effect on the genetic diversity of jack mackerel. In an allozyme study of orange roughy, Smith, Francis and McVeagh (1991) argued that fishing pressure was directly responsible for a loss of heterogeneity in that species.

10. OBJECTIVE 5

To use restriction enzyme analysis of mtDNA to survey the amount and type of genetic diversity in rainbow trout and Atlantic salmon in Australia.

Tasmanian salmonid species were sampled for genetic analysis in 1987 and 1998 (Atlantic salmon, n = 32; rainbow trout, n= 75; brown trout, n = 27 and brook char, n = 18). It had been planned to also obtain samples from mainland

Australia and maybe New Zealand. In the event, this work was wound back as requested in 1988 and investigations were limited to the Tasmania material which had already been collected.

It was demonstrated that all the 32 Atlantic salmon had identical mitochondrial genomes. The absence of variation in the mitochondrial genome was attributed to the transitory but significant reduction in brood stock numbers in the Gaden hatchery from which the Tasmanian population was derived.

A similar absence of variation was found in the 75 mitochondrial genomes analysed for rainbow trout. due probably to the repeated cycles of hatchery propagation of this species in Tasmania.

Work on the brown trout and brook char was ancillary to the objective of this study and was supported by University funds. Again no variation was found in the brown trout and only two variants were present in the brook char populations.

The results of this part of the study have been published in Ovenden, Bywater and White (1993) which is reprinted in Appendix D of this report.

11. OBJECTIVE 6

To determine if the mitochondrial DNA genome can be used as a genetic marker in selective breeding programs of cultured trout and salmon.

No work had been initiated towards this objective when we were requested to constrain out project mainly to the orange roughy. Accordingly, no data are available. It is, however, our opinion that, on the basis of results present above in relation to Objective 5, that it is very unlikely that it will be possible to effectively utilize the mitochondrial DNA genome as a genetic marker in

breeding programs of either rainbow trout or Atlantic salmon.

12. PUBLICATIONS

Six papers emanating from this project were published in international journals. In addition, a Master of Science thesis, based mainly on work carried out for this project, was successfully by Adam Smolenski. The papers and thesis are listed below. Copies of the papers are reproduced in the Appendices; one copy of the thesis accompanies this report. All publishable work arising from this project is now available in journals; there are no unpublished results. The FIRTA/FIRDC funding was acknowledged in all publications.

- **Ovenden, J.R., Smolenski, A.J. & White, R.W.G. 1989.** Mitochondrial DNA restriction site variation in Tasmanian populations of orange roughy (*Hoplostethus atlanticus*), a deepwater marine teleost. *Australian Journal of Marine and Freshwater Research* **40**, 1-9. (Appendix A)
- **Ovenden, J.R. 1990.** Mitochondrial DNA and marine stock assessment: a review. *Australian Journal of Marine and Freshwater Research* **41**, 835-853. (Appendix B)
- **Ovenden, J.R., Bywater, R. & White, R.W.G. 1992.** A computer program for aiding in the mapping of mitochondrial DNA restriction sites. *Journal of Heredity* **83**, 240-241. (Appendix C)
- **Ovenden, J.R., Bywater, R. & White, R.W.G. 1993.** Mitochondrial DNA nucleotide sequence variation in four species of salmonids (*Salmo salar, S. trutta, Oncorhynchus mykiss and Salvelinus fontinalis*) from Tasmania, Australia. *Aquaculture* **114**, 217-227. (Appendix D)
- **Smolenski, A.J. 1991.** *Mitochondrial DNA variation in orange roughy* (Hoplostethus atlanticus) *and jack mackerel* (Trachurus declivis). Master of Science thesis, University of Tasmania, Hobart.
- **Smolenski, A.J., Ovenden, J.R. & White, R.W.G. 1993.** Evidence of stock separation in southern hemisphere orange roughy (*Hoplostethus atlanticus,*

Trachichthyidae) from restriction enzyme analysis of mitochondrial DNA. *Marine Biology* **116**, 219-230. (Appendix E)

• **Smolenski, A.J., Ovenden, J.R. & White, R.W.G. 1994.** A preliminary investigation of mitochondrial DNA variation in southeastern Australian jack mackerel (*Trachurus declivis*, Carangidae). *Australian Journal of Marine and Freshwater Research* **45**, 495-505. (Appendix F)

13. CO-OPERATING INSTITUTIONS AND ACKNOWLEDGEMENTS

Many institutions, agencies and individuals contributed to this project by the collection of samples and the provision of advice.

We gratefully acknowledge the following people for their assistance in the collection of samples and for their helpful advice: Dr Jeremy Lyle, Dr Howell Williams, John Kitchener, Sean Riley and Grant Pullen (Tasmanian Division of Sea Fisheries): Cathy Bulman, Dr Nick Elliot and Dr Bob Ward (CSIRO Division of Fisheries Research); Gina Newton and Neil Klaer (Department of Primary Industry Bureau of Rural Resources); Steve Burnell and Danny Turner (South Australian Department of Fisheries); Dr Peter Smith (New Zealand Ministry of Agriculture and Fisheries); Ken Graham (New South Wales Fisheries Research Institute); Dr Andrew Payne and Rob Leslie (Department of Environment Affairs in Capetown, South Africa). Thanks also to 'Octopus Rose' Schwertfeger for assistance with the collection of jack mackerel samples from Eaglehawk Neck and to Neville Barrett and Jeremy Austin for collecting orange roughy samples on the FRV Soela cruises.

Specimens for the salmonid study were provided by the Inland Fisheries Commission of Tasmania, Sevrup Fisheries Pty Ltd, Penguin Seafoods Pty Ltd and Aquatas Pty Ltd.

14. OTHER BENEFITS

The FIRTA/FIRDC funds were used exclusively to operate this project. However, by contributing to the overall infrastructure of the laboratory other benefits were gained. These include a one year project FIRDC-funded project (Project 88/41 - Pilot study of larval recruitment and genetic variation of southern rock lobster populations), the results of which were published in Brasher, Ovenden, Booth and White (1992), Brasher, Ovenden and White (1992), Ovenden, Brasher and White (1992) and Ovenden and Brasher (1993) and a study of ice fish stock structures in the Southern Oceans, part of which has been published (Williams, R., Smolenski, A.J. & White, R.W.G. 1994).

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16. APPENDICES

A. **Ovenden, J.R., Smolenski, A.J. & White, R.W.G. 1989.** Mitochondrial DNA restriction site variation in Tasmanian populations of orange roughy (*Hoplostethus atlanticus*), a deepwater marine teleost. *Australian Journal of Marine and Freshwater Research* **40**, 1-9

B. **Ovenden, J.R. 1990.** Mitochondrial DNA and marine stock assessment: a review. *Australian Journal of Marine and Freshwater Research* **41**, 835-853.

C. **Ovenden, J.R., Bywater, R. & White, R.W.G. 1992.** A computer program for aiding in the mapping of mitochondrial DNA restriction sites. *Journal of Heredity* **83**, 240-241.

D. **Ovenden, J.R., Bywater, R. & White, R.W.G. 1993.** Mitochondrial DNA nucleotide sequence variation in four species of salmonids (*Salmo salar, S. trutta, Oncorhynchus mykiss and Salvelinus fontinalis*) from Tasmania, Australia. *Aquaculture* **114**, 217-227.

E. **Smolenski, A.J., Ovenden, J.R. & White, R.W.G. 1993.** Evidence of stock separation in southern hemisphere orange roughy (*Hoplostethus atlanticus*, Trachichthyidae) from restriction enzyme analysis of mitochondrial DNA. *Marine Biology* **116**, 219-230.

F. **Smolenski, A.J., Ovenden, J.R. & White, R.W.G. 1994.** A preliminary investigation of mitochondrial DNA variation in southeastern Australian jack mackerel (*Trachurus declivis*, Carangidae). *Australian Journal of Marine and Freshwater Research* **45**, 495-505. (Appendix F)

APPENDIX A

Ovenden, J.R., Smolenski, A.J. & White, R.W.G. 1989. Mitochondrial DNA restriction site variation in Tasmanian populations of orange roughy (*Hoplostethus atlanticus*), a deepwater marine teleost. *Australian Journal of Marine and Freshwater Research* **40** 1-9.

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Mitochondrial DNA and Marine Stock Assessment: A Review

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Contents

Introduction

To a fisheries manager, the most useful definition of a stock of marine animals is one that has a sound genetic base. Without knowledge of the number of interbreeding populations contained within an exploited species, management policies may not achieve long-term conservation goals. For example, the homogeneous exploitation of a fisheries resource composed of numerous unidentified interbreeding populations is likely to lead to either the serious erosion of population structure or the localized, and possibly permanent, extinction of a section of the resource. In this case, the prior application of genetic analyses to the fishery might have shown that it was composed of discrete populations that were experiencing little immigration. My definition of a stock is the largest group of animals that can be shown to be genetically connected through time. However, estimates of a marine stock can be made without a genetic component. For example, a stock is often defined as those animals occupying a physical region. Other estimates of management units are made assuming similarity in form. Animals with the same meristic or morphometric measurements are often assumed to constitute a stock (see Waldman *et al.* 1988, for example).

The pivotal role of genetic connectedness in the definition of fisheries stocks and the general availability of molecular genetic techniques have created a new scientific field: DNA characterization of marine animal populations for stock assessment (Avise 1985; Ferris and Berg 1987). At present, the least mysterious part of the animal genome, DNA molecules

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in the mitochondrion, is the target of molecular analysis for stock assessment purposes. This review provides an insight into this field for readers with a general scientific background. It consists of a description of how mtDNA (mitochondrial DNA) analysis is used for marine stock assessment, a summary of existing knowledge about the mitochondrial genome in marine species, and an evaluation of the usefulness of the technique in fisheries management.

The Mitochondrial Genome

Physical Properties

Large molecules of deoxyribonucleic acid (DNA) are found in the nuclei of cells. The genetic code they carry is contained in the linear order of four types of residues that form part of the DNA molecule. These are called nucleotides and are structurally classified as purines (adenine and guanine) or pyrimidines (cytosine and thymine). The nucleotide order, or sequence, is interpreted into the different types of proteins essential for organismal structure and function by the transcription and translation apparatus of the cell.

In animal eucaryotic cells, a small amount of DNA is found outside the nucleus within organelles in the cytoplasm. Mitochondria each contain about 10 identical copies of the mitochondrial genome. The genome is comparatively small (16 000 to 19 000 nucleotides), double-stranded, and circular. The genes it contains specify essential subunits of components of the respiratory pathway as well as the transfer and ribosomal RNA needed to manufacture those protein subunits. The genome contains very little non-functional DNA (Avise *et al.* 1987*a*; Moritz *et al.* 1987).

The nucleotide sequence of the mtDNA of two animals chosen at random from the same species is likely to be different. The difference may be in either the type or the number of nucleotides at a given location in the genome. The former are called point mutations and are likely to be the replacement of one nucleotide by its structural equivalent (Brown *et al.* 1982). The latter are called length mutations and are more common in a non-coding sequence (Cann and Wilson 1983; Hauswirth and Clayton 1985). Flexibility in the genetic code ensures that about one-third of the molecules with point mutations are tolerated by the cell because they have a minimal effect on the production of gene products or on replication. If a tolerated mutant genome is produced in a germ-line cell, vegetative segregation in successive germ-line cell divisions (Birky *et al.* 1983, 1989) makes it highly likely that all the mitochondria in the new individual arising from that germ cell will contain the mutant mtDNA.

Distribution in Space and Time

Animal mtDNA is thought to be strictly maternally inherited (Dawid and Blackler 1972; Giles *et al.* 1980). The results of at least two experimental tests are consistent with this; one study used the tobacco budworm (*Heliothus* spp.; Lansman *et al.* 1983) and the other used freshwater fish (*Poeciliopsis* spp.; Avise and Vrijenhoek 1987). In both cases, hybrids were produced between two strains having different mtDNA. As predicted, first-generation hybrids had the mtDNA characteristic of the maternal strain. For numerous generations, female hybrids were mated to the paternal strain. The hybrid offspring from these backcrosses always had the mtDNA from the original maternal strain. It was inferred that the small numbers of mitochondria in the sperm head that are injected into the ovum during fertilization do not survive in the cytoplasm of the zygote. The case for maternal mtDNA inheritance must remain open, however, as inheritance patterns amongst some *Drosophila* hybrids can be explained by a paternal mtDNA contribution (Satta *et al.* 1988).

The distribution of mitochondrial genomes in natural populations through time can be explained by analogy with male surname inheritance in human populations. Most animals possess different mitochondrial genomes in the same way that most humans have different surnames. However, within a generation, or across several generations, people or animals

may possess the same surname or mitochondrial genome. Possession of the same surname normally indicates a close familial, and thus genetic, relationship assuming that surname changes due to marriage are excluded. Animals possessing the same mtDNA are also assumed to be genetically similar. As with surnames, some animals may possess the same mtDNA yet are distantly related. These are regarded as convergent evolutionary events, and much effort is expended by geneticists to identify and exclude them.

The surname composition of each human generation depends, in part, on the number of families successfully raising male offspring to reproductive age and on the number of those offspring who marry and reproduce. It is similar for the mtDNA composition of natural populations. Maternal inheritance dictates that the mixture of mitochondrial lineages passed to the next generation depends on the relative reproductive success of each female. In addition, the number of individuals with different mitochondrial genomes in each generation can be augmented by the recruitment of animals with new mitochondrial genomes. This can occur by migration or *de novo* mutation. These new lineages are perpetuated within a population when females possessing the novel mitochondrial genomes contribute offspring to the next generation. In the same way as human surnames, mtDNA lineages become extinct when families leave no offspring.

If an impervious barrier to migration or intermarriage is imposed on an imaginary human population, random surname extinction and acquisition in each isolated group over numerous generations will ensure that the surname composition of each group becomes different. Differences in mtDNA between isolated animal populations also accumulate in this way. The degree of mtDNA difference between completely isolated groups can be used to estimate the amount of time since the groups were last part of an interbreeding population. Dating population divergence in this way requires that the rate of mtDNA evolution be accurately calibrated. I advise caution in using this approach as the rate of mtDNA evolution has not been calibrated in most taxa and published rates vary by orders of magnitude (Nei 1987, pp. 86–7). The use of mtDNA as an evolutionary 'clock' also assumes that populations of the species have been relatively free of bottlenecks, localized extinctions,

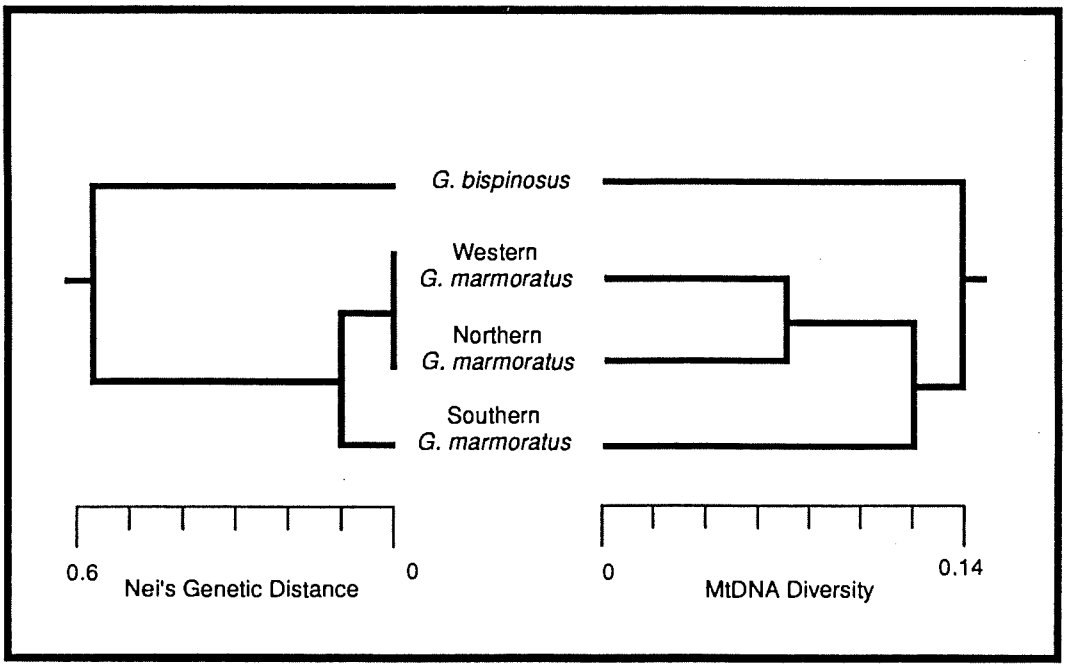

Fig. 1. Phylogenetic relationships of the freshwater fish species *Gadopsis marmoratus* as inferred from similarities in allozymes (Sanger 1986) and mtDNA (Ovenden *et al.* 1988).

and founder events. These assumptions may never be able to be validated, especially for marine species. If the animal groups with different mtDNA compositions are only partially isolated from one another, then the amount of difference between them is theoretically related to the number of migrants being mutually exchanged each generation.

Divergence in mtDNA nucleotide sequences accumulates more rapidly between populations than does divergence in nuclear DNA sequences. For example, Sanger (1986) found no allozyme frequency differences between western and northern Victorian populations of the river blackfish (*Gadopsis marmoratus*) at 21 nuclear gene loci. Yet sequence diversity of mtDNA from the two populations was 6·4% (Ovenden *et al.* 1988; Fig. 1). This ability of mtDNA to discriminate between closely related populations has been attributed to an increased rate of DNA mutation in the mitochondrial genome compared with the nuclear genome (Brown *et al.* 1979) as well as to a smaller effective gene flow between subdivided populations for mitochondrial genes compared with nuclear genes (Birky *et al.* 1989).

Application of mtDNA to Stock Assessment

Planning the Study

The objective of an mtDNA stock assessment study is firstly to reveal differences in the mtDNA of animals collected throughout the range of a species. Those differences are then used to estimate the size and number of partially or fully reproductively isolated populations making up that species. The sampling regime should include animals from populations that have been chosen to represent the extremes of hypothesized barriers to dispersal: both past and current, complete and incomplete. Professional advice should be sought at the commencement of a project about the sample sizes needed to provide a statistically significant test of stock structure hypotheses.

Restriction endonucleases are used to distinguish between the different types of mitochondrial genomes from animals included in the study. There are about 100 types of these enzymes, each derived from a different species of bacterium (Watson *et al.* 1987). Each enzyme breaks or digests DNA strands at a unique four- to six-nucleotide sequence called a restriction site. If the mtDNA from two animals belonging to the same species is incubated with the same restriction enzyme, it is likely that the enzyme will make the same number of breaks in each sample. This indicates that the mtDNA from both animals shares the same restriction sites for that enzyme and hence shares the same nucleotide sequence at the position of the restriction sites.

Occasionally, a restriction enzyme will not make the same number of breaks in the mtDNA from two animals. This is detected by counting and measuring the number of DNA fragments after enzyme digestion. The mtDNA from this pair of animals probably differs by at least one restriction site and hence by at least one nucleotide substitution. By using up to 10 or 20 different restriction enzymes, a picture can be built of the amount by which the mtDNA of each sampled animal differs from every other. Each type of mtDNA discovered amongst the samples is called a mitochondrial haplotype.

It is possible, but not essential, to plot the relative positions of restriction sites on a circular diagram, or map, of the mtDNA molecule. The inclusion of restriction-site mapping as part of an mtDNA stock assessment study most fully utilizes the data collected. It provides information about genome size and, with large sample sizes, region-specific rates of gene evolution. For example, one quadrant of the mitochondrial genome of the spotted mountain trout (*Galaxias truttaceus*) was found to be free of restriction-site polymorphism and was assumed to contain highly conserved ribosomal RNA genes (Ovenden and White 1990).

To analyse conspecific mtDNA for stock assessment studies, restriction enzymes must be sought that have more than one restriction site in the target DNA and whose restriction sites vary from individual to individual. These are called informative enzymes. For some species, the amount of restriction-site variation between the mitochondrial genomes of

individuals is small. These genomes are likely to be identical at most nucleotide positions. Informative enzymes for analysing very similar mitochondrial genomes are most likely to be those whose restriction sites consist of less than six, possibly four, consecutive nucleotides. As short restriction sites are more common than longer ones, enzymes with short restriction sites digest or break the DNA of individuals more frequently than enzymes with longer restriction sites. Consequently, enzymes with restriction sites four nucleotides long effectively sample more of the nucleotide sequence of the genome than enzymes with restriction sites composed of six or eight nucleotides. Thus, these enzymes are more likely to detect differences between the genomes under analysis. Enzymes with restriction sites longer than four nucleotides can be used in stock assessment studies if the nucleotide sequence of the mitochondrial genomes is more diverse.

The number of different restriction enzymes used in marine stock assessment studies is crucial to the production of statistically significant results. Haplotype frequency differences between collection localities is affirmative evidence for genetic partitioning. However, if fish with the same haplotype are found in more than one collection locality, the localities are not necessarily connected genetically. Haplotypes may also be shared by collection localities because insufficient numbers of informative restriction enzymes were used to analyse the mtDNA of fish from each locality. The number of informative enzymes that should be used to minimize false conclusions is directly proportional to the magnitude of mtDNA sequence diversity in that species. Sequence diversity can be estimated according to the method of Nei and Li (1979) or Nei and Tajima (1983) from the relative occurrence of 10 to 15 randomly chosen types of restriction sites in the mtDNA of about 20 randomly chosen individuals from the species under study. It is advisable to estimate sequence diversity in a pilot study before the start of a stock assessment study so that the number of restriction enzymes needed to efficiently and accurately survey haplotype frequencies among collection localities is known in advance. The pilot study presents a good opportunity to determine which tissue is the most suitable for mtDNA recovery and to determine the best collection and storage conditions.

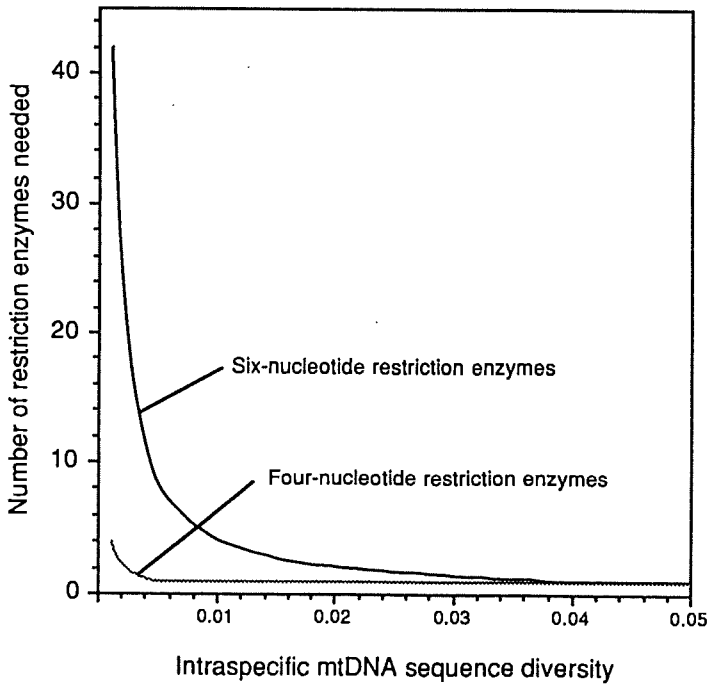

Fig. 2. Minimum number of restriction enzymes that should be used to adequately survey haplotype frequencies in species with varying amounts of mtDNA sequence diversity. The derivation of the relationship is explained in the text. Genome size is taken to be 16 500 nucleotide pairs.

Once an estimate of intraspecific sequence diversity has been calculated, the minimum number of enzymes to use in a stock assessment study can be approximated. Assuming that nucleotides occur randomly at each position in the DNA molecule with a frequency of 0·25, a unique six-nucleotide restriction site occurs every 4096 (4^6) nucleotides. A four-nucleotide site occurs every 256 nucleotides (4^4). If the genome size in the target species is 16 000 nucleotides long, there would be 3·91 six-nucleotide and 62·50 four-nucleotide sites per genome. If the mean amount of intraspecific diversity is 0·002 substitutions per nucleotide, each genome sampled varies on average by 32 (0·002 × 16 000) nucleotides at random throughout the genome. Each enzyme with a six-nucleotide restriction site surveys 23·46 (3·91 × 6) nucleotides per mtDNA molecule. The probability that one enzyme with a six-nucleotide restriction site will detect at least one variant nucleotide is 0·046 ((32 × 23·46)/16 000). As the aim is to detect at least one variant nucleotide in every genome sampled, at least 22 ($0{\cdot}046^{-1}$) enzymes with six-nucleotide restriction sites or two enzymes with four-nucleotide sites would be necessary. If a population displays little diversity (less than 0·0015, for example), it is more practical to use enzymes with four-nucleotide restriction sites instead of those with six (Fig. 2). However, if enzymes with four-nucleotide restriction sites are used when diversity is high, every animal may be found to have a different haplotype. Enzymes with six-nucleotide restriction sites are normally used in this situation to facilitate subsequent analyses.

Laboratory Work

In the laboratory, mtDNA is isolated from liver, heart, spleen or ovary tissue collected within 1 h of death. It is crucial to store tissue immediately upon death as DNA yields otherwise decline markedly with increasing time after death. Tissue can be stored in liquid nitrogen for up to 2 years before use. Some types of tissue can be stored in TEK buffer (50 mM Tris, 10 mM EDTA, 1·5% (w/v) potassium chloride; pH 7·5) for up to 10 days before use. Alternatively, extraction can be done with tissue from freshly killed animals.

After buffered tissue lysates have been enriched for mitochondria by differential centrifugation, mtDNA is recovered by extraction with buffer-equilibrated phenol followed by alcohol precipitation (Chapman and Powers 1984). Alternatively, mtDNA can be purified from tissue lysates by using isopycnic caesium chloride gradients (Lansman *et al.* 1981; Darley-Usmar *et al.* 1987). Each gram of tissue yields about 100 μL of aqueous mtDNA, of which 1–3 μL are needed for each restriction-enzyme digest. The addition of bovine serum albumin (DNase-free, 100 μg mL^{-1}) and spermidine (1 mM) can enhance the completeness of mtDNA digestion. Digested fragments of mtDNA are radioactively tagged in an enzymic reaction (Ovenden *et al.* 1989) so that their position in an agarose or polyacrylamide gel matrix after electrophoresis can be determined with X-ray film. The state of a restriction site in the mtDNA of each fish is inferred from the size and number of DNA fragments after restriction-enzyme digestion (fig. 1 in Ovenden *et al.* 1989).

Analysis of Results

To assess the stock composition of the species sampled, the mtDNA relationships between groups of animals need to be established. Animals can be grouped into classes according to geographical, temporal or biological similarity. If there is a discernible pattern to the mtDNA relationships between these classes, and if the pattern is consistent with known features of the biology of the species, then it may be tentatively concluded that the species is composed of more than one interbreeding population, or stock. However, the lack of a discernible mtDNA pattern does not indicate the lack of genetically discrete populations. In this situation, it is always possible to argue that distinct populations are present in the species but that their presence is not reflected in the mitochondrial genetics of the species. For example, the cryptic populations may have diverged so recently that the sorting of mitochondrial haplotypes between them has not yet occurred (Avise *et al.* 1987*a*).

A χ^2 test can determine whether the frequencies of haplotypes in each class of animals

are significantly different. However, the number of haplotypes found in an mtDNA study is often large and the distribution of individuals between the haplotypes is often skewed, resulting in low sample numbers per cell, which can invalidate the χ^2 analysis (Cochran 1954). This problem can be overcome by comparing the magnitude of the χ^2 value obtained with the magnitudes of many (1000) pseudo-χ^2 values obtained from random rearrangements of the data table. A way to perform these random rearrangements while maintaining equal row and column totals is presented by Roff and Bentzen (1989).

The quantitative information possessed by each haplotype can be used for stock assessment by calculating the amount of nucleotide sequence diversity (Nei and Li 1979; Nei and Tajima 1983) among animals within each class and among animals in different classes. If the classes represent genetically discrete populations, then the amount of diversity within each class will be less than the amount of diversity between each class. This can be assessed statistically by first correcting the magnitude of interclass diversity for intraclass 'noise' (equation 25 in Nei and Li 1979) and then testing whether the corrected interclass diversity is significantly different from zero. This latter test can be performed by making use of the standard error of interclass diversity as calculated by the method of Nei and Jin (1989).

An alternative method for detecting the presence of genetic subdivision amongst the classes of animals is to perform a gene diversity analysis (Nei 1973; Chakraborty and Leimar 1987). The magnitude of the G statistic (G_{st}) is interpreted as the relative amount of total diversity that is due to the presence of partially or fully reproductively isolated populations amongst all of the animals studied. This approach has been adapted to mtDNA data by Takahata and Palumbi (1985), who use restriction sites as alternate alleles, and by Birky *et al.* (1989), who use mitochondrial haplotypes as alternate alleles. The significance of the G_{st} value calculated from restriction sites can be tested by comparing its magnitude with the magnitudes of a large number of G_{st} values obtained from random rearrangements of the data that preserve the original class sizes (Palumbi and Wilson 1990). The significance of the haplotype G_{st} value can be evaluated against a series of jacknifed G_{st} values obtained by omitting one class in turn.

If the presence of discrete stocks within the target species is indicated by any of the foregoing analyses, then it may be possible to determine the evolutionary relationship between them. Phylogenies can be constructed from the presence or absence of restriction sites or fragments by using computer packages (PHYLIP, version 3.2, Felsenstein 1989; PAUP, version 2.4, D. L. Swofford, Illinois Natural History Survey, Champaign, Illinois; MacCLADE, version 2.1, W. Maddison and D. Maddison, Harvard University, Cambridge, Massachusetts). For fully reproductively isolated populations, a phylogeny constructed between the haplotypes of each stock may reveal features of the mitochondrial genome that are characteristic of each stock (see, for example Saunders *et al.* 1986 and Avise *et al.* 1987*a*). Phylogenies may also be constructed from matrices of numerical similarity (normally sequence diversity) between haplotypes or individuals. The unweighted pair-group method with arithmetic means (UPGMA) is the technique most commonly used (Nei 1987, p. 293). A way of computing standard errors for branching points in these types of evolutionary trees is given by Nei *et al.* (1985).

If it is decided that the stocks are only partially reproductively isolated, then the amount of gene flow between them can be estimated (Takahata and Palumbi 1985; Chakraborty and Leimar 1987; Slatkin 1989) under a model of island populations at equilibrium (Wright 1943). It is important to remember that gene flow is defined as the average numbers of immigrants per generation that reproduce successfully in their adopted population. It does not include immigrants that are transient visitors only.

Characteristics of mtDNA in Marine Species

Invertebrates

Vawter and Brown (1986) and Palumbi and Wilson (1990) both measured intraspecific

mtDNA variation in the North American sea urchin, *Strongylocentrotus purpuratus*. Their estimates of intraspecific mtDNA sequence divergence were 0·0099 and 0·0048 respectively. Palumbi and Wilson (1990) found no subdivision of mtDNA haplotypes between the eastern and western coasts of North America. They did find subdivision between *S. droebachensis* sampled from eastern and western North America. The mean intraspecific divergence for this species, including samples from both coasts, was 0·001.

The mtDNA of the deep-sea scallop (*Placopecten magellanicus*) is 34 000 nucleotides long, about twice the metazoan average (Snyder *et al.* 1987). This scallop genome appears to be at the upper limit of size for non-yeast eucaryotic mtDNA, a position also occupied by the 26 000-nucleotide mtDNA of the parasitic nematode *Romanomermis culicivorax* (Powers *et al.* 1986). The mtDNA of scallops collected from the same geographical area fell into one of seven size classes, each varying by a multiple of 1200 nucleotides (La Roche *et al.* 1990). Of the 300 scallops assayed, 7% possessed mtDNA from more than one size class. The inheritance of the extensive size heteroplasmy of *P. magellanicus* mtDNA must be examined in more detail before sequence variation in this mitochondrial genome can be used for stock assessment. It is unclear at present whether size heteroplasmy develops during the lifetime of an individual (Linnane *et al.* 1989) and is therefore an acquired rather than inherited trait. The occurrence of size heteroplasmy is fairly common (see citations in Rand and Harrison 1989).

Edwards and Skibinski (1987) reported no size heteroplasmy in mitochondrial genomes from mussels (*Mytilus edulis* and *M. galloprovincialis*). They sampled 187 individuals from five localities in south-western England where these species are known to hybridize. Using three six-nucleotide restriction enzymes known to recognize polymorphic sites, they found 24 mtDNA haplotypes. The frequency of the four most common haplotypes was significantly different in four out of seven χ^2 tests between localities. Although no more sophisticated tests of subdivision were performed with the data, the study appears to show that mussel haplotypes are geographically subdivided along the coastline of south-western England.

An extreme case of genetic subdivision has been reported for another mollusc, the American oyster *Crassotrea virginica* (Reeb and Avise 1990). In all, 82 haplotypes were distinguished with 13 restriction enzymes amongst 212 individuals from 14 localities on the eastern coast of the United States from the Canadian border to Texas. Individual oysters fell into two distinct genetic classes corresponding to localities either within the Gulf of Florida or on the remainder of the Atlantic coast. Mean sequence divergence between haplotypes in either of these classes was a large 2·6%.

Genetic subdivision is not a general feature of geographically defined populations of molluscs. No pattern was detected in mtDNA restriction fragments within or among 68 blacklip abalone (*Haliotis rubra*) collected from four approximately equidistant locations around the coastline of Tasmania (Barrett 1989). As two recent non-genetic analyses of the population biology of this species have proposed a limited amount of larval dispersal, it was concluded that the amount of gene flow between adult populations in the form of larval movement may be large enough to homogenize the mitochondrial genetics of the species.

Batuecas *et al.* (1988) confirmed that the order of genes in the mtDNA of *Artemia*, an arthropod, was similar to that of the completely sequenced mtDNA of *Drosophila*, another arthropod. Sequence variation in the mtDNA of natural populations of the spiny lobster *Panulirus argus* (Komm *et al.* 1982; McLean *et al.* 1983) and the horseshoe crab *Limulus polyphemus* (Saunders *et al.* 1986) has been estimated on the eastern coast of the United States. Both studies on *P. argus* reported distinctive restriction haplotypes from Key West, on the tip of the Florida peninsula, compared with haplotypes about 110 km (Komm *et al.* 1982) and 280 km (McLean *et al.* 1983) to the east along the Florida coastline. However, the low numbers of animals (24 and 24) and restriction enzymes (2 and 6) used in these two studies, and the general lack of statistical analyses performed, provide only a preliminary glimpse of genetic subdivision in this species. Sequence variation and geographic subdivision of mtDNA in the southern hemisphere rock lobster complex (*Jasus* spp.) is being analysed at present (Ovenden, D. J. Brasher and R. W. G. White, unpublished data).

Sequence variation of mtDNA in *Limulus polyphemus* is a spectacular example of geographical partitioning (Saunders *et al.* 1986). The horsehoe crab has a linear distribution along the inshore Atlantic coast of North America and has free-swimming trilobite larvae. Ten mtDNA haplotypes were distinguished with 12 restriction enzymes amongst 99 individuals sampled from 15 localities extending from Panama City, Florida, in the south to Dover Point, New Hampshire, in the north. Three haplotypes were only found to the north of southern Georgia. The remaining seven haplotypes were found only to the south of that point. A minimum of nine mutations separated the haplotypes in the north and south. Saunders *et al.* (1986) note that the location of the genetic break point coincides with a transition zone between warm-temperate and tropical faunal groups. The transition point also corresponds to the location of the genetic transition between two genetically distinct classes of oysters (*Crassotrea virginica*; Reeb and Avise 1990).

Vertebrates

There are similarities in the type of mtDNA variation found in two North American anadromous fish species. The American shad (*Alosa sapidissima*) spawns in eastern coastal rivers. Juveniles spend 4–6 years at sea before homing to their natal rivers to spawn. The striped bass (*Morone saxatalis*) has a similar life history and distribution. Among 43 striped bass, Wirgin *et al.* (1989) found three fish with a mean genome size of 18 002 nucleotide pairs, three fish with a mean genome size of 17 775 nucleotide pairs, 25 fish with a mean genome size of 17 633 nucleotide pairs, and 12 fish with a mean genome size of 17 551 nucleotide pairs. Bentzen *et al.* (1988) found that shad mtDNA also occurred in size classes that were 18 300 (214 fish) and 19 800 (30 fish) nucleotide pairs in length. The basis for the size variation of shad mtDNA was a variable number of 1500-nucleotide repeated sections of DNA.

Wirgin *et al.* (1989) further distinguished between striped bass genomes according to the presence or absence of restriction sites recognized by two restriction enzymes (*Xba*I and *Rsa*I). Bentzen *et al.* (1988) scored shad mtDNA for the presence or absence of restriction sites recognized by *Sal*I and *Kpn*I. Striped bass mtDNA haplotypes were completely subdivided into Gulf of Mexico *v.* Atlantic coast populations (Wirgin *et al.* 1989). Shad haplotypes were not convincingly subdivided between Atlantic coast freshwater drainages (Bentzen *et al.* 1988), although a reanalysis of the data (Roff and Bentzen 1989) has shown them to be so.

American eels (*Anguilla rostrata*) and European eels (*Anguilla anguilla*) spawn in the western tropical mid-Atlantic Ocean. Juveniles are transported throughout the North Atlantic by ocean currents. Avise *et al.* (1986) assayed intraspecfic mtDNA sequence variation in non-spawning adults collected from the western coast of North America (*A. rostrata*) and from England and Ireland (*A. anguilla*). No evidence was found for genetic subdivision in either species. The mtDNA of the two species was shown to be different with 11 of the 14 restriction enzymes used.

Avise *et al.* (1987*b*) set out fo find genetically subdivided marine species and chose the catfish and the toadfish as likely candidates because of their lack of a larval phase. The catfish species, *Bagre marinus* and *Arius felis*, inhabit coastal waters on the eastern coast of North America. The males incubate batches of eggs in their mouths, and after 8–11 weeks juveniles emerge to colonize the adult habitat. The toadfish, *Opsanus beta* and *O. tau*, are sedentary coastal species, also without a pelagic larval phase. Amongst *A. felis*, 11 mtDNA haplotypes were identified. The most common haplotype, found in 45 of the 60 individuals, was evenly distributed across the collection localities. Likewise, the most common *B. marinus* haplotype (7 out of 12 individuals) was widely distributed. The five *O. tau* haplotypes formed two groups of two or three haplotypes each. Each group possessed a common haplotype, and the two groups were characterized by the presence or absence of a restriction site recognized by *Stu*I. The groups also had characteristic collection localities to either the north or the south of Beaufort, North Carolina, where individuals belonging

to either group were collected. In the Gulf of Mexico, *O. beta* haplotypes were grouped into eastern or western clades characterized by the possession, or loss, of a *Pst*I restriction site.

Genetic subdivision of mtDNA haplotypes between collection localities has not yet been reported for the pelagic species skipjack tuna (*Katsuwonus pelamis*; Graves *et al.* 1984), albacore tuna (*Thunnus alalunga*; Graves and Dizon 1989), South African hake (*Merluccius capensis* and *M. paradoxus*; Becker *et al.* 1988), Atlantic herring (*Clupea harengus*; Kornfield and Bogdanowicz 1987), deep-sea orange roughy (*Hoplostethus atlanticus*; Ovenden *et al.* 1989), and Atlantic cod (*Gadus morhua*; Smith *et al.* 1989). However, as intraspecific divergence in these species was low (0·19% for the orange roughy, for example), and as mtDNA was assayed with six-nucleotide enzymes, it is possible that haplotypes were scored as shared by groups of animals because insufficient numbers of restriction sites were assayed. For stock assessments to be made of species such as these, it may be routinely necessary to assay the mtDNA with enzymes recognizing restriction sites composed of four nucleotides.

In extreme contrast to all other marine fish species, two menhaden species (*Brevoortia tyrannus* and *B. patronus*) have been found to have an intraspecific mtDNA sequence diversity of 2·4% (Avise *et al.* 1989). Along with a species of iguanid lizard that has a mean mtDNA sequence diversity of 1·5%, these fish species have the highest intraspecific

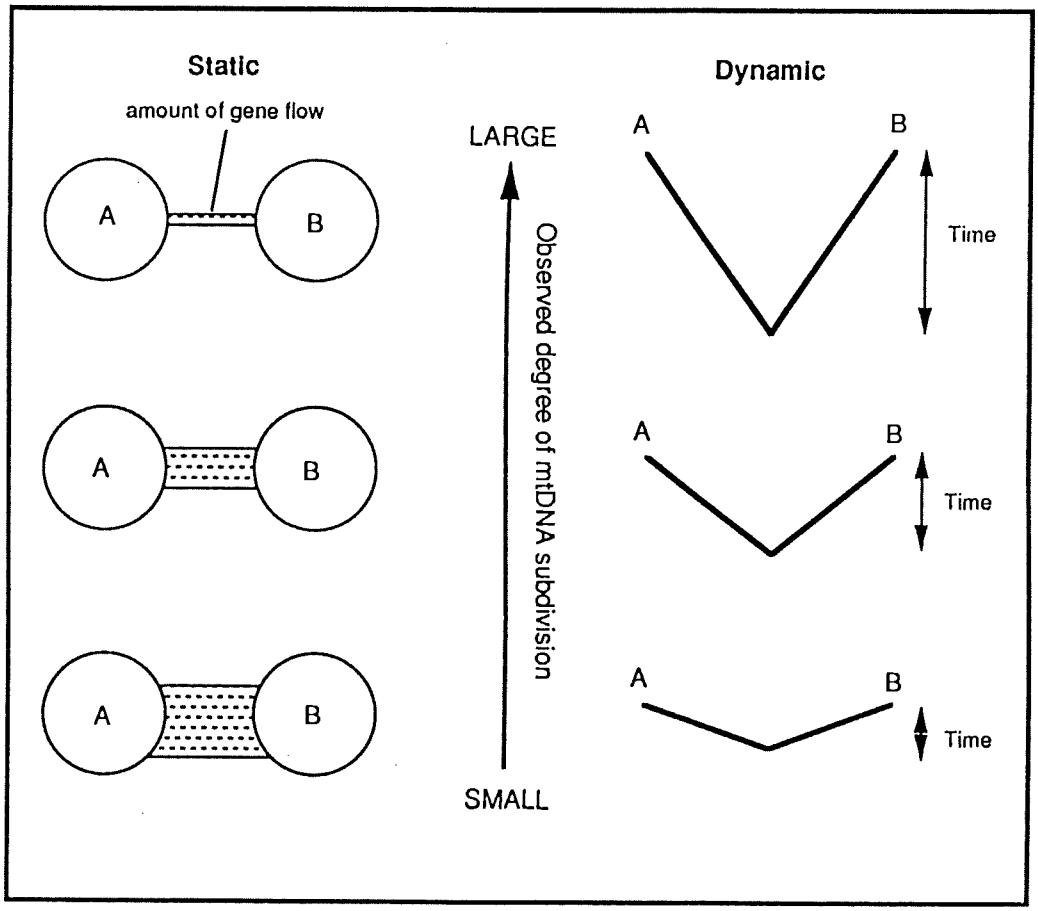

Fig. 3. Static and dynamic interpretations of the evolution of populations *A* and *B*, which are genetically subdivided to a greater or lesser extent. A large amount of subdivision between populations *A* and *B* can be explained by either a small amount of gene flow between them (static interpretation) or a remote common ancestor (dynamic interpretation). Alternatively, if populations *A* and *B* are weakly subdivided, they may either be experiencing large amounts of gene flow (static interpretation) or have a recent common ancestor (dynamic interpretation).

diversity of vertebrate species assayed so far. The authors have not yet presented an analysis of menhaden haplotypes that would allow an assessment of the degree of geographic subdivision.

Interpretation of mtDNA Nucleotide Sequence Variation in Marine Species

Two Perspectives on Observed Genetic Variation

Sequence variation in the mitochondrial genome has been found to be geographically subdivided in some marine species. These include marine invertebrates such as sea urchins (*Strongylocentrotus droebachensis*; Palumbi and Wilson 1990), mussels (*Mytilus edulis* and *M. galloprovincialis*; Edwards and Skibinski 1987), American oysters (*Crassotrea virginica*; Reeb and Avise 1990), spiny crayfish (*Panulirus argus*; Komm *et al.* 1982; McLean *et al.* 1983), and horsehoe crabs (*Limulus polyphemus*; Saunders *et al.* 1986). Marine vertebrates such as striped bass (*Morone saxatalis*; Wirgin *et al.* 1989) and toadfish (*Opsanus tau* and *O. beta*; Avise *et al.* 1987*b*) have also been found to be genetically subdivided.

The observation of genetic subdivision within a species is an instantaneous view of time of the complex process of evolution. There are two evolutionary interpretations of observed genetic variation (Fig. 3). The first interpretation assumes that, from our viewpoint in time, evolution within the species is static or at equilibrium. The second interpretation is that the observed genetic variation is part of a dynamic process that has not come to equilibrium. It is impossible to adopt one perspective without acknowledging the existence of the other.

Accordingly, observed mtDNA subdivision within a species can be explained by the existence of a partial reproductive barrier between subdivided groups across which gene flow is at equilibrium. This interpretation is an appealing and frequent explanation of genetic subdivision between marine populations. However, genetic subdivision may indicate the past imposition and continued existence of a total reproductive barrier between the subdivided groups. In this case, genetic variation within groups may be in a state of flux in response to the presence of the barrier.

Despite the assumed homogenizing effect of the ocean, complete barriers to reproduction do exist in the marine environment as they are a prerequisite for speciation. The perceived nature of marine reproductive barriers is limited by our terrestrial imaginations. Some obvious examples include the formation of land bridges by falling sea levels (Bassian Isthmus; Galloway and Kemp 1981) or rising continents (Isthmus of Panama; Vawter *et al.* 1980). *Strongylocentrotus droebachensis*, the sea urchin species shown to be genetically subdivided between the eastern and western coast of North America by Palumbi and Wilson (1990), may have become reproductively isolated by a geographical barrier. Less discernible barriers may involve sharp thermoclines, nutrient-poor currents, or gyres. For example, genetically distinct populations of sessile invertebrates may be totally reproductively isolated because they occur along coastlines that experience a differential increase and decrease of water temperature throughout the year. If spawning occurred at a particular water temperature, adults in one part of the cline could not interbreed with adults in another. Add to this scenario larval settlement that occurs only at a certain water temperature, and a complete reproductive barrier may be maintained within the species despite its potential for a large amount of larval dispersal.

Magnitude and Nature of mtDNA Sequence Variation

Mean mtDNA sequence diversity is the average nucleotide diversity between each pair of individual mitochondrial genomes in a population. Estimates of its size vary by about three orders of magnitude for marine species and encompass the entire range of terrestrial intraspecific diversity (Avise *et al.* 1989). The species having the most diverse mitochondrial genome (*Brevoortia* spp., 2·4%; Avise *et al.* 1989) and the least diverse mitochondrial genome (*Morone saxatalis*, 0·004%; Wirgin *et al.* 1989) are marine. However, it is more common for marine species to have lower intraspecific mtDNA sequence diversities (less than 1·0%, for example) than terrestrial species.

Table 1. Summary of studies on the mitochondrial DNA of marine species

The magnitude of intraspecific divergence is the mean divergence between all pairs of individuals. In a few cases (*), it is the mean divergence between pairs of haplotypes. n.d., Not determined; n.a., not applicable since the species has experienced translocation by humans

Species	Magnitude of intraspecific divergence (%)	Number and type of restriction enzymes used	Number of individuals studied	Range of haplotype frequencies	Any genetic subdivision observed?	Reference
			Phylum Echinodermata			
Stronglylocentrotus purpuratus	0·48	11; 5–6-nucleotide 2; 4-nucleotide	38	0·026–0·237	No	Palumbi and Wilson (1990)
Strongylocentrotus droebachensis	0·11	11; 5–6-nucleotide 2; 4-nucleotide	41	0·024–0·537	Yes	Palumbi and Wilson (1990)
Strongylocentrotus purpuratus	0·99	5; 4-nucleotide	16	n.d.	n.d.	Vawter and Brown (1986)
Asterina pectinifera	Nucleotide sequence and gene order determined for 3849-nucleotide-pair fragment					Himeno *et al.* (1987)
			Phylum Mollusca			
Placopecten magellanicus	Genome twice the normal size of metazoan mtDNA					Snyder *et al.* (1987
Mytilus edilus/ galloprovincialis complex	n.d.	3; 6-nucleotide	187	0·02–0·33	Yes	Edwards and Skibinski (1987)
Crassotrea virginica	2·6*	12; 6-nucleotide 1; 4-nucleotide	212	0·005–0·321	Yes	Reeb and Avise (1990)
			Phylum Arthropoda			
Panulirus argus	n.d.	2; 6-nucleotide	24	n.d.	Provisional	Komm *et al.* (1982)
Panulirus argus	n.d.	6; 6-nucleotide	24	n.d.	Provisional	McLean *et al.* (1983)
Artemia spp.	Sequencing and southern plotting used to investigate gene order					Batuecas *et al.* (1988)
Limulus polyphemus	n.d.	12; 6-nucleotide	99	0·010–0·485	Yes	Saunders *et al.* (1986)

		Phylum Chordata				
Order Anguilliformes						
Anguilla rostrata	0·1*	11; 5–6-nucleotide 3; 4-nucleotide	109	0·009–0·670	No	Avise *et al.* (1986)
Anguilla anguilla	n.d.	11; 5–6-nucleotide 3; 4-nucleotide	29	0·015–0·212	No	Avise *et al.* (1986)
Order Clupeiformes						
Clupea harengus	1·66*	7; 6-nucleotide	69	0·014–0·174	No	Kornfield and Bogdanowicz (1987)
Alosa sapidissima	n.d.	10; 6-nucleotide	244	n.a.	Provisional	Bentzen *et al.* (1988)
Brevoortia tyrannus	2·4	18; 6-nucleotide	17	0·030–0·061	n.d.	Avise *et al.* (1989)
Brevoortia partronus	2·4	18; 6-nucleotide	16	0·030–0·061	n.d.	Avise *et al.* (1989)
Order Siluriformes						
Arius felis	1*	13; 6-nucleotide 1; 4-nucleotide	60	0·017–0·750	No	Avise *et al.* (1987*b*)
Bagre marinus	1*	13; 6-nucleotide 1; 4-nucleotide	12	0·083–0·583	No	Avise *et al.* (1987*b*)
Order Gadiformes						
Gadus morhua	n.d.	11; 6-nucleotide 1; 4-nucleotide	35	n.d.	No	Smith *et al.* (1989)
Merluccius capensis	1·3*	11; 6-nucleotide	26	0·038–0·231	No	Becker *et al.* (1988)
Merluccius paradoxus	0·57*	11; 6-nucleotide	24	0·042–0·542	No	Becker *et al.* (1988)
Order Batrachoidiformes						
Opsanus tau	1*	13; 6-nucleotide 1; 4-nucleotide	53	0·019–0·472	Yes	Avise *et al.* (1987*b*)
Order Beryciformes						
Hoplostethus atlanticus	0·19	10; 6-nucleotide	49	0·020–0·735	No	Ovenden *et al.* (1989)
Order Perciformes						
Morone saxatalis	0·004	5; 5–6-nucleotide 5; 4-nucleotide	43	n.a.	Yes	Wirgin *et al.* (1989)
Trachurus declivis	n.d.	11; 6-nucleotide	59	0·017–0·780	n.d.	A. J. Smolenski, J. R. Ovenden and R. W. G. White (unpublished data)
Katsuwonus pelamis	n.d.	9; 6-nucleotide	16	n.d.	No	Graves *et al.* (1984)
Thunnus alalunga	n.d.	10; 5–6-nucleotide 2; 4-nucleotide	23	n.d.	No	Graves and Dizon (1989)

There are several explanations for the low amount of mtDNA sequence diversity observed in marine species. Mitochondrial genome evolution may be generally slower in some marine animals. Variation in the rate of genome evolution, both mitochondrial and nuclear, has often been reported (Nei 1987). Intraspecific mtDNA diversity in some marine species could be low because these species have experienced recent bottleneck events (Nei *et al.* 1975). Such phenomena have been shown to reduce mtDNA diversity (Wilson *et al.* 1985; Ovenden and White 1990). The marine species with low levels of mtDNA sequence diversity are a disparate assemblage, ranging from North American inshore sea urchins (*Strongylocentrotus purpuratus*; Palumbi and Wilson 1990) to anadromous fish (*Morone saxatalis*; Wirgin *et al.* 1989) and pelagic fish (*Hoplostethus atlanticus*; Ovenden *et al.* 1989). The cause of bottleneck events in these species could be biogeographical or epidemiological (O'Brien and Evermann 1988).

Another explanation for low mtDNA sequence diversity in some marine species could be family-specific mortality. This type of mortality is most likely to occur in species in which the offspring of individual females are grouped into egg-masses or schools. The death of these groups of siblings (due to encounters with unfavourable microhabitats or predators, for example) would eliminate the genetic contribution of some females to the next generation. An extreme form of family-specific mortality could occur when only a small proportion of females contributes offspring to the next generation. The high fecundity of some marine species could ensure stable population sizes in the face of a large amount of family-specific mortality. Geographical or temporal aspects of spawning behaviour may preclude the formation of maternally related groups of offspring in some species. In these species, family-specific mortality may not occur. Experimental investigations of the occurrence of family-specific mortality in high-fecundity compared with low-fecundity species with differing spawning behaviours are needed. Family-specific mortality could be responsible for a low amount of mtDNA sequence diversity within species since, at each spawning season, a relatively large proportion of females does not contribute its haplotypes to the next generation. The extent to which family-specific mortality could depress mtDNA sequence diversity in species with large population sizes and multiple spawnings per female has yet to be determined.

Of the marine species assayed to date, few have equal haplotype frequencies across geographic classes. The sea urchin (*Strongylocentrotus purpuratus*; Palumbi and Wilson 1990), the European eel (*Anguilla anguilla*; Avise *et al.* 1986), the South African hake (*Merluccius capensis*; Becker *et al.* 1988), and the menhaden (*Brevoortia* spp.; Avise *et al.* 1989) come close with haplotype frequencies between 0·02 and about 0·30 (Table 1). Other species have highly skewed haplotype frequencies, with the frequency of the most common haplotype being 0·78 (*Trachurus declivis*; A. J. Smolenski, J. R. Ovenden and R. W. G. White, unpublished data), 0·75 (*Arius felis*; Avise *et al.* 1987*b*), and 0·74 (*Hoplostethus atlanticus*; Ovenden *et al.* 1989) (Table 1). Assuming that mtDNA diversity was adequately surveyed with an appropriate number of restriction enzymes in each of these studies highly skewed haplotype frequencies may have been caused by a past decrease in effective population size or by selection acting upon haplotypes. It is thought that mitochondrial haplotypes are susceptible to selection (MacRae and Anderson 1988), but no conclusion has been reached about the characteristics of the selective process.

Conclusion

It was predicted, on the basis of mtDNA sequence diversity in populations of terrestrial species, that the mitochondrial genome would be ideal for identifying reproductively subdivided populations of marine species (Avise 1985; Ferris and Berg 1987). Several years later, this goal has been realized for only 6 of the 15 studies in which an unequivocal result was obtained (Table 1). The cases in which mtDNA has not revealed the presence of separate stocks may truly reflect the absence of genetic subdivision within the populations.

However, in some cases sample sizes were not large enough to obtain a statistically significant result (Komm *et al.* 1982; McLean *et al.* 1983; Graves *et al.* 1984; Graves and Dizon 1989; Table 1) or insufficient restriction enzymes were used to survey genome variation (Komm *et al.* 1982). Although not successful in every case, the application of mtDNA analysis to a number of species has revealed the presence of genetically discrete stocks when other stock assessment techniques have failed to do so.

The magnitude of mtDNA sequence diversity is critical to the success of a stock assessment program; subdivision can be detected only if the mtDNA molecule is found to be variable within a species. Marine species are unusual in that some of them appear to have less variable mitochondrial genomes than previously studied terrestrial and freshwater species. To a certain extent, this problem can be overcome by knowing the mtDNA diversity of a species in advance and by using a sensitive sequence assay, namely restriction enzymes that recognize restriction sites composed of four, not six, nucleotides. Explanations of this phenomenon may arise when estimates of mtDNA diversity have been made in a larger number of marine species.

The frequencies of mtDNA haplotypes between classes of samples have been found to be skewed. As with low mtDNA sequence diversity, this problem may be overcome when researchers use more sensitive techniques to assay variation between individuals. The unusual nature of this data has been overcome to a certain extent by the use of bootstrapping procedures and other methods that place confidence limits on summary statistics. Measures of statistical variation are essential in genetic stock assessment studies for accurate interpretation of the results obtained and widespread acceptance of the conclusions drawn. I hope that more advanced analysis techniques will be forthcoming as the field expands.

It is up to the researcher to infer whether the subdivided populations identified by mtDNA analysis are totally reproductively isolated or are experiencing small amounts of gene flow. This can be best achieved by a consideration of all known aspects of the biology of the species. It is important to remember that estimates of gene flow take into account only the number of individuals exchanged between populations during each generation that successfully contribute genes to the next generation. Large numbers of non-breeding transient migrants may also be present within the genetically defined stock that were not sampled during the study. The presence of these individuals can be detected only with a more thorough sampling programme or by non-genetic techniques. If it is decided that the populations are totally reproductively isolated, then unique features of the mtDNA from each population can be used to assign unknown individuals from widely dispersed localities to their stocks of origin.

On the horizon of marine population genetics is a new DNA analysis tool, the polymerase chain reaction. This innovative method could be used to produce large quantities of mitochondrial or nuclear DNA from marine individuals whose small body size or state of preservation precludes the use of present DNA analysis techniques. The polymerase chain reaction makes use of the capacity of DNA polymerase to synthesize double-stranded DNA from minute quantities of single-stranded DNA taken from each individual. The single-stranded DNA is first allowed to bond with much shorter single strands of DNA called primers. A heat-stable form of DNA polymerase then catalyses the synthesis of double-stranded DNA from the single-stranded template. The newly synthesized double-stranded DNA is then denatured by heat into single strands, and the cycle is repeated. Each cycle results in a 100% increase in the amount of target DNA so that, after 20–30 cycles, the target DNA predominates in the reaction mixture (Erlich 1989).

The entire mitochondrial genome is probably too large for amplification with the polymerase chain reaction. However, known sections of the genome can be targeted with so-called 'universal primers' (Kocher *et al.* 1989). If the marine animal populations being studied are thought to be closely related, then the target DNA can be chosen from amongst the regions of the mitochondrial genome that are known to have the most variable

nucleotide sequence. The amplified target DNA from each animal can then be analysed with restriction enzymes in the conventional manner, or its entire nucleotide sequence can be determined.

The availability of DNA in workable quantities from small marine animals will expand the field of marine population genetics to include planktonic organisms. Information from such studies could be of great use in the study of fishery recruitment patterns. For example, if it had been previously established that spawning populations of a particular species were genetically discrete, then polymerase chain reactions could be used to determine the parental origin of individual pre-settlement larvae. A similar approach could be used to track the fate of post-settlement recruits. If the DNA of newly recruited animals did not match that of the existing adult population, the post-settlement selection of some sort might be operating.

An mtDNA analysis laboratory is expensive to establish and maintain. Capital items, including high-speed refrigerated centrifuges, electrophoresis apparatus, and good-quality laboratory fittings, may cost $100 000. The most expensive recurrent costs are restriction enzymes, radioactive nucleotides, photographic film for autoradiographs, and electrophoresis ingredients such as ultra-pure agarose, polyacrylamide, and Tris buffer. An allowance of $15 000 a year should be made for these consumable items. The laboratory also needs well-trained staff, who ideally need to be zoology, genetics or biochemistry graduates.

The use of mtDNA in stock assessment studies is expensive and pioneering. However, the technique has already assisted in the identification of previously cryptic subpopulations of marine animals. With a greater knowledge of how the genome evolves in marine species, and with an understanding of the technique by fisheries managers, mtDNA analysis will become an indispensible aid to fisheries management.

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APPENDIX B

Ovenden, J.R. 1990. Mitochondrial DNA and marine stock assessment: a review. *Australian Journal of Marine and Freshwater Research* **41**, 835-853.

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Mitochondrial DNA Restriction Site Variation in Tasmanian Populations of Orange Roughy (*Hoplostethus atlanticus*), a Deep-water Marine Teleost

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Abstract

The orange roughy, *Hoplostethus atlanticus*, forms the basis of an important trawl fishery in Australia and New Zealand which is currently being over-exploited. To gain information about the stock structure of an Australian orange roughy population, ten restriction enzymes were used to survey the restriction-site variation in the mitochondrial genome of 23 individuals collected from the east coast and 26 individuals collected from the west coast of Tasmania. The mean diversity between all 49 genomes was a low 0·19%. Of the eleven haplotypes identified, only one was present in both the east and west coast samples. Four haplotypes were found only in the east coast sample and six were found only in the west coast sample. Eight haplotypes were related to the haplotype found on both coasts by the gain or loss of one restriction site. One haplotype collected in the east coast sample was related to one other east coast haplotype by one site change. One haplotype collected from the west coast was related to a west coast haplotype by one site change. If this pattern is confirmed by further mitochondrial DNA studies, it could be inferred that gene flow between the orange roughy populations on the east and west coasts of Tasmania is low.

Extra keyword: stock assessment.

Introduction

The orange roughy, *Hoplostethus atlanticus* (Trachichthyidae), is found on the continental slope surrounding south-eastern Australia and New Zealand, in the Atlantic Ocean and in the Mediterranean Sea (Last *et al.* 1983). The species is an important resource for the southern Australian and New Zealand deep-sea trawl fishery. The fishery takes advantage of large, dense aggregations of adults from which large catches can be made. The presence of these aggregations is unexplained, but it is thought that they form from time to time in the same locality throughout the year (Williams 1987). Little is known about the larval or reproductive biology of the species.

In some localities, the catch rate of orange roughy is declining. This is thought to be a direct consequence of over-exploitation. To achieve the highest sustainable yield from this species, accurate information about all aspects of its population biology is needed. Of particular importance to fisheries management is the degree to which geographically defined populations of fish move and interbreed between localities. If there is a large amount of migration and/or interbreeding between localities the effect of localized overfishing is minimal. Conversely, overfishing can cause localized extinctions if gene flow between localities is low.

Estimates of genetic diversity between and within populations of marine animals have been used to identify reproductively isolated populations or stocks. The success, or otherwise, of these studies depends in part on the amount of genetic diversity displayed by the

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populations (Smith and Fujio 1982; Gyllensten 1985) and the degree to which it is subdivided between localities. For example, Shaklee and Salini (1985) showed that Australian barramundi (*Lates calcarifer*) populations from three widely separated regions in northern Australia had significantly different allele frequencies. They concluded that there were at least three stocks, or subpopulations, of barramundi in Australia. Smith (1986) applied similar techniques to southern Pacific and northern Atlantic Ocean populations of orange roughy but found only small amounts of genetic differentiation between them. He concluded that the genetic similarity of geographically distant populations may be maintained by migration between ocean ridges and sea mounts.

The findings of Smith (1986) contrast with a parasitological study by Lester *et al.* (1988). Lester *et al.* examined the parasite load of 1251 orange roughy collected from eight localities in southern Australia and three localities in New Zealand. From these eleven localities, five Australian and three New Zealand orange roughy stocks were identified. They concluded that orange roughy is a sedentary species with little movement of fish between localities.

We have chosen to use restriction-enzyme analysis of the mitochondrial genome to investigate the population dynamics of Tasmanian orange roughy. Certain features of the extra-nuclear mitochondrial genome, including its clonal inheritance between generations, its rapid rate of evolution and its conservative molecular structure, make it a powerful magnifying glass for the examination of fisheries stock composition (Avise 1985). The level of temporal and geographical heterogeneity in the nucleotide sequence of the orange roughy mitochondrial genome reported here adds to the body of data which is being amassed about the evolution of the mitochondrial genome in marine animals.

Table 1. Location, month of collection and numbers of *H. atlanticus* mitochondrial haplotypes sampled

Haplotype	East Coast	West Coast		
		May	August	Combined
AAAAAAAAAA	19	10	7	17
AAAAAAABAA	1	0	0	0
AAAAAABAAA	1	0	0	0
AAAAABAAAA	0	1	0	1
AAABAAAAAA	1	0	0	0
AABAAAAAAA	0	1	0	1
AAAABABAAA	1	0	0	0
AAAAACAAAA	0	0	2	2
AAAACAAAAA	0	1	0	1
AAACAAAAAA	0	1	0	1
AACAAAAAAA	0	1	2	3
Total	23	15	11	26

Materials and Methods

H. atlanticus individuals were collected from the west coast and the east coast of Tasmania by the CSIRO fisheries research vessel *Soela* using a 50-m, high-rise Engel bottom trawl. The fish were captured between 680 and 1320 m and were dead when brought to the surface. The east coast population was sampled in May 1987 within a 36-km radius of 41°28′S.,148°47′E. The west coast population was sampled twice: in May 1987 within a 0·9-km radius of 41°15·5′S.,144°02·4′E. and in August 1987 within a 3·4-km radius of 41°15·2′S.,143°59·8′E. Twenty-three and twenty-six fish, respectively, were chosen at random from each locality for analysis (Table 1). Ovary and/or liver tissue, weighing 0·95–4·99 g, was immediately removed from each fish and stored in liquid nitrogen. The fish were 34·6 ± 4·6 cm (mean ± s.d.) long (fork length, FL) and their weight was 1410 ± 400 g.

The method described by Chapman and Powers (1984) was used to extract the mtDNA from the fish tissue. One-microlitre aliquots of mtDNA were pipetted into 20 μL of the recommended digestion

buffer containing one of the following restriction endonucleases: *ApaLI* (recognition sequence: GTGCAC), *AvaI* (CPyCGPuG), *BanI* (GGPyPuCC), *BglI* (GCCNNNNNGGC), *BglII* (AGATCT), *HindIII* (AAGCTT), *NcoI* (CCATGG), *PvuII* (GAGCTG), *SacII* (CCGCGG), *XhoI* (CTCGAG) (New England Biolabs, California). Each digestion contained between 3·5 to 30 units of restriction-enzyme activity. The genomes were digested for about 2 h. Selected genomes were digested with 11 other restriction endonucleases. These digests produced either too many [*Eco0109* (PuGGNCCPy), *HinfI* (GANTC), *TaqI* (TCGA)] or too few [*BamHI* (GGATCC), *BclI* (TGATCA), *BstEII* (GGTNACC), *BstNI* (CCA/TGG), *MluI* (ACGCGT), *NdeI* (CATATG), *SalI* (GTCGAC) and *StyI* (CCA/TA/TGG)] fragments to yield informative results and were excluded from the analysis.

The mtDNA fragments were end-labelled with alpha-^{32}P-dCTP using the exonuclease and polymerase activity of Klenow fragment DNA polymerase I (Biotechnology Research Enterprises, South Australia). This technique differs from the standard method of Brown (1980) which uses the polymerase activity of the Klenow fragment only. We allowed the Klenow fragment DNA polymerase I to degrade the DNA in the 3′ to 5′ direction at 37°C in the absence of nucleotide triphosphates for 0·5 h. This step was immediately followed by strand reconstruction at room temperature in the presence of one radio-labelled and three unlabelled nucleotide triphosphates. This method of endlabelling maximizes the incorporation of labelled nucleotides.

Following ethanol precipitation and washing, the radiolabelled mtDNA fragments were loaded onto a vertical 1·4% agarose gel as previously described (Ovenden *et al.* 1988). Size standards were provided on each gel by bacteriophage lambda DNA (New England Biolabs, CA.) digested with *HindIII*. The gels were dried and autoradiographed overnight. For all ten restriction enzymes used to digest the mtDNA, each variant fragment pattern was given an upper-case letter from A to C. Each orange roughy genome was described by a ten-letter summary of these designations (Table 1). Each different ten-letter summary was assumed to represent a unique mitochondrial haplotype. A circular diagram showing the location of restriction enzyme cleavage sites was constructed for each haplotype from the numbers and sizes of restriction fragments generated.

The number of restriction sites shared per genome pair and the average number of sites per genome were used to calculate ∂, the mean number of base substitutions per nucleotide, using the maximum likelihood approach of Nei and Tajima (1983) which takes into account the variable size of restriction sites. This method of calculating diversity assumes that the four types of nucleotides are randomly arranged in the DNA sequence and evolutionary change of the DNA sequence occurs solely by random nucleotide substitution (Nei and Tajima 1983). Although it has been suggested that the composition and evolution of mtDNA violates these assumptions (Adams and Rothman 1982), Nei and Tajima (1983) conclude that these violations affect the outcome of the calculations only when the divergence between the genomes is greater than 0·3 mutations per base pair. This is more than three orders of magnitude larger than the diversity estimates reported here.

Results

DNA fragment patterns on autoradiographs were easier to interpret if the DNA was derived from ovary as opposed to liver tissue. Oocytes are a rich source of mitochondria (Pikó and Taylor 1987). Nucleic acids in the metabolically active liver may have been degraded during the time between fish death and dissection. An estimate of the size of the mitochondrial genome in *H. atlanticus* was obtained by averaging the sum of the sizes of all the fragments for each haplotype. The estimate was 16 211 ± 816 base pairs (mean ± s.d.). All orange roughy genomes were assumed to be the same size, as the standard deviation of the size estimate was low and variant fragment patterns were not consistently produced by any one genome.

Digestion with *ApaLI*, *AvaI*, *SacII* and *XhoI* identified no restriction site variation in the mitochondrial genome of *H. atlanticus*. The remaining enzymes identified 2 to 3 variant genomes each. Amonst the 49 orange roughy genomes, *NcoI* and *PvuII* identified two different fragment patterns each, and four enzymes, *BglI*, *BglII*, *HindIII* and *BanI*, produced three distinct fragment patterns each (Fig. 1).

The assignment of the presence or absence of restriction sites from fragment patterns was straightforward for all enzymes except *BglI*. The enzymes *BglII* and *HindIII* both produced two rare haplotypes, each of which, compared with the common haplotype, had

gained one restriction site. The rare haplotype (B) identified by *PvuII* differed from the common haplotype (A) by the loss of one site. One rare *BanI* haplotype (B) lost one restriction site, while the other *BanI* haplotype (C) had lost two sites compared with the common haplotype. *NcoI* identified one rare haplotype which varied from the common haplotype by the addition of one restriction site (Fig. 1).

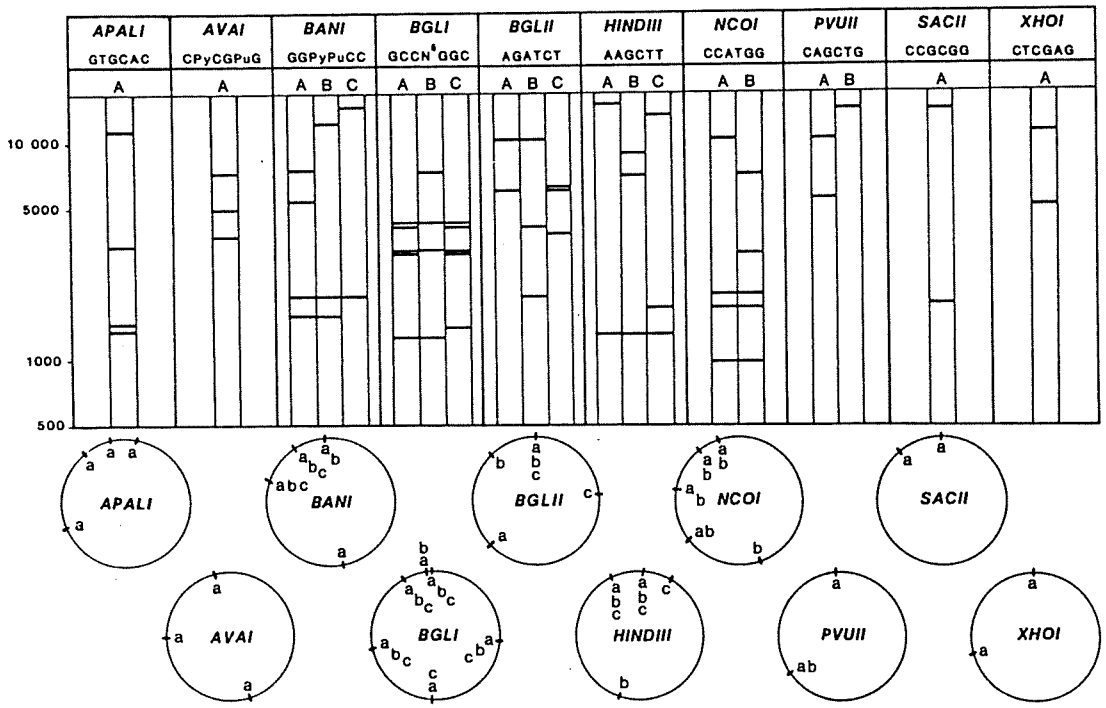

Fig. 1. Fragment patterns and restriction site diagrams for variant orange roughy mitochondrial haplotypes identified by ten restriction enzymes. Fragment sizes are shown in base pairs. The presence or absence of restriction sites is indicated on the circular diagrams using the same lettering system (*a*, *b*, *c*) as variant fragment patterns. The restriction sites have not been mapped, but placed arbitrarily on the circular diagrams.

The common *BglI* fragment pattern (A) was interpreted as having six restriction sites. Five restriction fragments were visible on autoradiographs but the smallest fragment, estimated to be less than or equal to 200 base pairs, could not be seen (Fig. 1). The presence of the 200 base pair fragment was proposed to account for a change in the mobility of the smallest visible fragment in the common haplotype compared with haplotype C. Thus B and C haplotypes are assumed each to have lost one site relative to the common haplotype. This interpretation of the *BglI* fragment patterns will be tested during the future construction of a map of the location of restriction sites.

Total genome diversity between all orange roughy individuals sampled was 0·0019 base substitutions per base pair. The diversity of the east coast individuals was 0·0012 base substitutions per base pair. The diversity of the west coast individuals was 0·0024 base substitutions per base pair. Because the overall genome diversity is about equal to the intra-populational genome diversity, we have not attempted to estimate mean interpopulational diversity between the east and west coasts.

Eleven mitochondrial haplotypes were identified amongst the 49 orange roughy individuals surveyed in this study (Table 1). The mean diversity between each haplotype was 0·0059 base substitutions per base pair. Nineteen out of 23 individuals sampled from the east coast and 17 out of 26 individuals sampled from the west coast had the same haplotype (AAAAAAAAAA). Four fish sampled from the east coast possessed four unique haplotypes while nine fish sampled from the west coast possessed another six unique haplotypes.

The west coast haplotypes were represented by one (four cases), two (one case) or three (one case) individuals each. Of the eleven haplotypes characterized in this study, only one (AAAAAAAAAA) was found in samples taken from both coasts. The frequency of these haplotypes does not differ significantly between samples taken from the east coast versus the west coast of Tasmania (Chi-square test on combined class frequencies, $\chi^2 = 5{\cdot}235$, $P = 0{\cdot}073$, d.f. = 2).

Each haplotype, except AACAAAAAAA and AAAABABAAA, is related to the common AAAAAAAAAA haplotype by a single site gain or loss (Fig. 2). Four haplotypes (AAAACAAAAA, AAAAABAAAA, AAAAACAAAA and AAAAAABAAA) have gained a *BglII*, *HindIII*, *HindIII* and *NcoI* site respectively. The remaining four haplotypes (AAABAAAAAA, AAAAAAABAA, AAACAAAAAA and AABAAAAAAA) have lost a *BglI*, *PvuII*, *BglI* and *BanI* site, respectively, compared with the common haplotype AAAAAAAAAA.

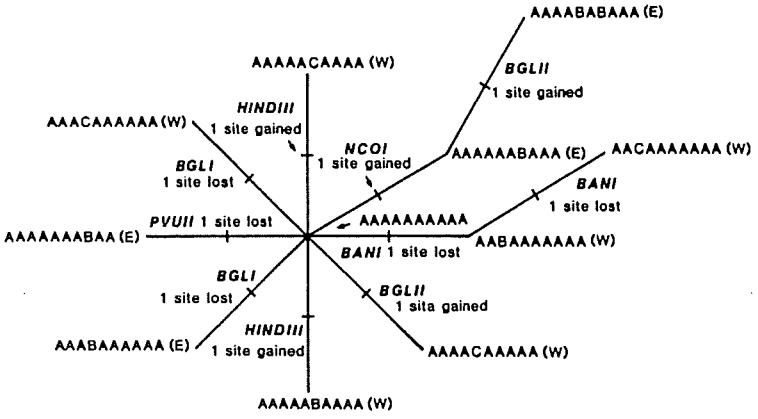

Fig. 2. Dendrogram showing the relationship of the common haplotype (AAAAAAAAAA) to rare haplotypes from populations from the west (W) and east (E) coast of Tasmania. The type of restriction site which is either gained or lost along each branch is indicated.

The two haplotypes AACAAAAAAA and AAAABABAAA which are not related to the common type by a single site gain or loss are related to two other rare haplotypes by a single site gain or loss. AACAAAAAAA is related to AABAAAAAAA by a *BanI* site loss and AAAABABAAA is related to AAAAAABAAA by a *BglI* site gain. These closely related haplotype pairs are found on the east coast or on the west coast of Tasmania, respectively.

Dicussion

This study contributes to a practical and theoretical framework which will enable restriction enzyme analysis of mtDNA to be used for stock identification of marine species. Restriction enzyme analysis estimates the relative amount of sequence diversity in the mitochondrial genome, and by inference genetic subdivision, between arbitrary or predetermined animal groupings. Under most circumstances, each haplotype is noramlly represented in the sample by more than one individual. Thus, intrapopulational diversity estimates between pairs of haplotypes are larger than diversity estimates between pairs of individuals. For example, the diversity between orange roughy haplotypes sampled from the west coast is 0·0054 mutations per base pair but the diversity between individuals in that sample is 0·0024 mutations per base pair. Similarly, the haplotype diversity of the eastern sample is 0·0050 whereas the individual diversity is 0·0012 mutations per base pair. Intrapopulational diversity estimates between individuals are often a starting point for further genetic analyses (Wilson *et al.* 1985; Ovenden *et al.* 1987). Estimates of diversity between haplotypes are useful when the phylogenetic relationships of the taxa are being investigated (Bermingham and Avise 1986; Avise *et al.* 1987*b*; Ovenden *et al.* 1988).

The amount of diversity which we observed between orange roughy mitochondrial haplotypes, 0·0054 base substitutions per base pair, is similar to the intraspecific diversity

estimates made between haplotypes of two species of North American marine catfish (Ariidae, 0·006) and between two species of North American marine toadfish (Batrachoididae, 0·006 and 0·009) (Avise *et al.* 1987*b*). In general, it appears that intraspecific mitochondrial DNA diversity for marine teleosts is lower than for non-marine species.

Although our estimates of orange roughy sequence diversity are based on 49 individuals only, theoretical studies have shown that, under certain circumstances, accuracy in the measurement of diversity is not increased by assaying large numbers of individuals. For example, Nei (1985) and Takahata and Nei (1985) have calculated that if the time since the divergence of populations is relatively small and the sample size is large, the standard error involved in the estimation of mtDNA diversity will be large because a large proportion of the variance is caused by stochastic factors. The small amount of sequence divergence (0·19%) discovered between mitochondrial genomes in the Tasmanian orange roughy population implies that, in future studies of the stock structure of this fishery using restriction enzyme analysis of mitochondrial DNA, sample sizes and the number of base pairs surveyed per genome will need to be large.

We have found preliminary evidence for the hypothesis of genetic subdivision of the Tasmanian population of orange roughy. If this hypothesis is confirmed by future genetic studies, it may indicate a low level of genetic interchange between the populations to the east and west of Tasmania. Although the deep sea has been described as globally homogeneous, there is no *a priori* reason why barriers to interbreeding can not exist between deep-sea populations of animals. Wilson and Hessler (1987) maintain that geographic variation followed by speciation will occur if the scale of spatial heterogeneity is large (or small) enough to effect populations. Avise *et al.* (1987*b*) have reported geographical localization of mitochondrial DNA haplotypes amongst populations of inshore marine toadfish (*Opsanus tau* and *O. beta*) along the coast either to the north or west of Florida, U.S.A. Populations of marine catfishes along the same coastline showed a random distribution of haplotypes. There were no detectable differences in the mtDNA of skipjack tuna (*Katsuwonus pelamis*) from the Atlantic and Pacific Oceans (Graves *et al.* 1984) or between American eels (*Anguilla rostrata*) collected along 4000 km of north American coastline (Avise *et al.* 1986). Homogeneity of eel mtDNA was explained by the presence of a single panmictic spawning population. The small numbers of tuna (n = 9, 6) which were analysed by pooling samples may have hidden mtDNA variation in the study by Graves *et al.* (1984).

The mitochondrial genomes of orange roughy sampled from the west coast population (0·24%) are more diverse than those sampled from the east coast population (0·12%). It is not possible to determine whether this result reflects population differences, as small sample sizes preclude statistical tests. If the magnitude of mitochondrial DNA diversity does differ between coasts, it may be explained by the two populations having a different temporal history of bottlenecks or rate of evolution of mtDNA nucleotide sequences. Other factors responsible may be differential rates of migration into or away from the populations, temporal variation in the composition of the populations, or different effective population sizes. At present, the west coast population may be larger in size; the commercial catch per unit effort is higher on the west coast than on the east coast (Williams 1987).

An analysis of orange roughy larval dynamics would test our working hypothesis of the existence of separate orange roughy stocks to the east and west of Tasmania by identifying potential barriers to gene flow. Pankhurst *et al.* (1987) report that orange roughy eggs are buoyant, but we are unaware of the collection of any orange roughy larvae from New Zealand or Tasmanian waters. We plan to continue our studies of orange roughy populations by examining variation in mtDNA sequences in larger numbers of individuals collected throughout the southern Pacific.

Smith (1986) reported only slight differentiation in the nuclear genome between orange roughy sampled from the Tasman Sea and the south-west Pacific Ocean and fish sampled in the north-east Atlantic Ocean, 21 000 km away. Allozymes at 13 of the 22 enzyme loci examined were fixed or were present in high frequencies in all samples. High-frequency

alleles at eight polymorphic loci were shared by the Tasman–Pacific and Atlantic samples. High-frequency alleles were not shared by the Tasman–Pacific and Atlantic samples at only one polymorphic locus, *Gpi-1*. *Gpi-1*^{a} was the most common allele in the Tasman–Pacific samples and *Gpi-1*^{b} was the most common allele in the Atlantic sample. Smith also observed that the distribution of alleles with low frequencies provided some evidence for regional variation amongst the populations sampled.

The average frequency of some of Smith's rare or private alleles, those which are found in only one of his seven sampled populations (*Aat*^{c}, *Gpdh-1*^{a}, *Gpdh-1*^{c}, *Gpdh-2*^{a}, *Gpdh-2*^{c}, *G6pdh*^{c}, *Idh-1*^{c}, *Idh-2*^{a}, *Ldh-1*^{a} and *Mdh*^{d}), can be used to compute an index of gene flow between populations, *Nm* (Slatkin 1985). *Nm* is equivalent to the average number of individuals exchanged between local populations per generation. Theoretical studies have shown that, if the effect of selection is disregarded, genetic drift through time will lead to differentiation between sub-divided populations unless the populations exchange about one migrant per generation. Using equation (3) from Slatkin (1985), where $a = -0{\cdot}505$, $b = -2{\cdot}440$, $P(1) = 0{\cdot}023$ and $N/25 = 3{\cdot}072$, *Nm* for Smith's orange roughy populations is 4·553. For another widely distributed marine fish, the milkfish *Chanos chanos*, *Nm* for 14 populations sampled across 10 000 km of the Pacific Ocean from the Philippines to Hawaii was 4·2 (Winans 1980; Slatkin 1985). Interpopulational electrophoretic variation at 38 loci was extremely low for the milkfish (Winans 1980), as it was for the orange roughy (Smith 1986). Despite the large distances separating local populations of orange roughy and milkfish, nuclear genetic similarity appears to be maintained by gene flow of the equivalent of about 4 to 5 immigrants per generation.

Our working hypothesis of the existence of separate orange roughy stocks to the east and west of Tasmania lends support to the parasitological study by Lester *et al.* (1988). They suggested that there are at least eight orange roughy stocks in Australian and New Zealand waters, in apparent contradiction to the study by Smith (1986). How should the results obtained by Smith (1986), Lester *et al.* (1988) and mitochondrial DNA analyses be evaluated to ensure the long-term survival of the Tasmanian orange roughy fishery? Allozyme electrophoresis has been used extensively in fisheries management to test the null hypothesis that all samples have been drawn from a single interbreeding population and therefore should have equal allozyme frequencies (Shaklee 1983). While rejection of the null hypothesis positively identifies a sub-divided stock, its acceptance does not positively identify a single interbreeding stock. Smith (1986) found an above-average level of genetic heterozygosity in orange roughy populations but was unable to reject the null hypothesis that the samples were drawn from a single interbreeding unit. If, however, the orange roughy resource is managed as a single unit and local populations are overfished to extinction, the calculated amount of gene flow between them (*Nm* being 4–5 individuals per generation) would probably be insufficient to re-establish viable populations. We believe that the mtDNA and parasitological results are highly relevant to the management of the resource.

Lester *et al.* (1988) state that, whereas the use of parasites as markers provides information about the immediate history of individual fish, genetic studies provide information about the long-term history of the population. They argue that under at least two circumstances, recent or incomplete population subdivision, the use of parasites to unravel fisheries stock structure is superior to genetic analysis. We believe that this comment applies only to nuclear gene analysis, and that mtDNA analysis is just as useful as, if not better than, parasite analysis in these circumstances. The rapid rate of mtDNA sequence evolution ensures that mitochondrial genes remain heterogeneous between populations which are exchanging so many migrants that the allozyme gene frequencies are homogenized (Avise *et al.* 1987*a*). Thus, mitochondrial gene differences may remain static in populations experiencing nuclear gene flow. The rapid rate of mtDNA evolution also ensures sequence diversity between populations which have recently been reproductively subdivided. MtDNA has the added advantage over parasite analysis of being able to reflect the recent history of numerous, maternally related generations of fish. Provided there is no evidence for

differential sex-specific migration, then mtDNA analysis may provide the highest genetic resolution possible at the population level.

Our initial impression of orange roughy is of a species with low vagility, with individuals occupying the same geographic area throughout their lifetimes. If this hypothesis is not falsified it has major implications for the management of the resource, as local populations could easily be extinguished by overfishing and would not be replenished by migration from other areas.

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APPENDIX C

Ovenden, J.R., Bywater, R. & White, R.W.G. 1992. A computer program for aiding in the mapping of mitochondrial DNA restriction sites. *Journal of Heredity* **83**, 240-241.

A Program for the Estimation of Restriction Endonuclease Site Positions from Restriction Fragment Size and Number: An Aid for Mitochondrial DNA Analysis

J. R. Ovenden, R. Bywater, and R. W. G. White

Mitochondrial genome sequence polymorphism is being extensively used to study the operation of evolution within populations and species. Patterns of relatedness between individuals are normally described in terms of phylogenetic trees and genetic distances. Restriction site, rather than restriction fragment, data are more accurate for this purpose. For example, the behavior of restriction fragments among individual genomes violates the assumption of character independence necessary for cladistic analysis, as the presence of a fragment in an individual is accompanied by the absence of two or more fragments whose additive size equals that of the gained fragment. Similarly, genetic distance is likely to be overestimated, as fragments are much less likely to be shared by pairs of individuals than are restriction sites.

The conversion of a restriction fragment profile for the mtDNA of a particular individual into a restriction site map can be accomplished using the double digestion technique (Ausubel et al. 1990; Maniatis et al. 1982). Once a map of the relative location of restriction sites is made for one individual, it can be used as a reference for the determination of site presence or absence in the mtDNA from a series of other individuals from their restriction fragment profiles. Sequence variation in the entire data set can then be analyzed using restriction site, rather than restriction fragment, similarity. Our program, SITEFINDER, is an interactive tool that uses the rapid calculation power of the personal computer to facilitate the deductive decisions needed to construct a circular restriction map using the double digestion technique.

The mapping procedure is initiated by determining the relative position of sites recognized by three restriction enzymes. These enzymes are chosen so that their sites can be unambiguously located solely from the sizes of fragments produced by single and reciprocal double digests. In practice, most sets of three enzymes, which recognize only one, two, or three sites each, lead to unambiguous solutions. The user must perform the appropriate digestion experiments in the laboratory and then use the program to produce the initial map.

Sites recognized by further enzymes can be located progressively on the map using information about the size of fragments produced by double digests between the new enzyme and at least two enzymes whose sites have been previously mapped. The program assists in constructing the site map by orienting single enzyme site maps according to the number and size of fragments produced by double digests, reports on the number of possible solutions, and displays intermediate and final maps as circular diagrams. The program is designed for use by an operator who understands the process of constructing restriction maps from double digest information. However, novices will also find the program and accompanying operating instructions useful.

At any stage during map building, the sites recognized by all or some of the mapped enzymes can be displayed graphically and numerically on the screen. The accuracy of the map can be checked by comparing the known sizes of double digest fragments to the sizes calculated by the program from the positions of the mapped sites. When map construction is complete, the program can be used to store the site position information in a way that allows quick and easy access for the design of subsequent experiments.

Data are entered progressively during the operation of the program and are saved in a file compatible with most simple text editors, such as QUED or EDIT. The results, in terms of site positions clockwise from a reference point, can be printed on the imagewriter and also stored in text format. The program is a compiled application written in Microsoft BASIC for the Macintosh (512, Plus, SE, and II).

To receive the program and operating instructions, send a blank, initialized, 3.5-inch, double-sided diskette to Dr. J. R. Ovenden. The source code is also available on request.

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APPENDIX D

Ovenden, J.R., Bywater, R. & White, R.W.G. 1993. Mitochondrial DNA nucleotide sequence variation in four species of salmonids (*Salmo salar, S. trutta, Oncorhynchus mykiss and Salvelinus fontinalis*) from Tasmania, Australia. *Aquaculture* **114**, 217-227.

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Mitochondrial DNA nucleotide sequence variation in Atlantic salmon (*Salmo salar*), brown trout (*S. trutta*), rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) from Tasmania, Australia

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ABSTRACT

The unique properties of the mitochondrial genome were used to investigate features of the population dynamics and origins of the species. No restriction site variation was found among samples of Tasmanian Atlantic salmon or among rainbow trout mitochondrial genomes. This lack of mtDNA diversity in Atlantic salmon may have been caused by a transitory, but significant, decrease in brood stock numbers in the hatchery from which the Tasmanian population was derived. Rainbow trout may have low levels of mtDNA diversity due to repeated cycles of hatchery and wild propagation in New Zealand, mainland Australia and in Tasmania. A freshwater and a sea-running population of brown trout were found to have identical mitochondrial genomes, despite the introduction to Tasmania of numerous strains of northern hemisphere fish, each of which could logically be assumed to carry its own characteristic mtDNA. In combination with historical evidence, it is suggested that only the first introduction of a 'few hundred' brown trout in 1864 produced acclimated populations and that the small number of maternal lines in this founding population accounts for the observed lack of mtDNA diversity. Two brook trout haplotypes were found among a sample of only 18 Tasmanian fish, suggesting that the founding stock of brook trout which was introduced to Tasmania in 1962 possessed considerable mtDNA diversity despite its supposed hatchery origin.

INTRODUCTION

The unique properties of nucleotide sequence polymorphism of mitochondrial DNA (mtDNA) can provide glimpses into the recent evolutionary history of animal populations (Avise et al., 1987). The extra-nuclear genome is relatively small (16–19 000 nucleotide pairs), maternally inherited without recombination at meiosis (Lansman et al., 1983; Avise and Vrijenhoek, 1987)

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and has been observed to have a relatively rapid rate of mutation across generations (Avise et al., 1987). Identical copies of the genome are found within all of the mitochondria of every individual, except for a few rare cases (Moritz et al., 1987). The mechanics of mtDNA sequence evolution ensure that interbreeding animal populations, which are partially or fully reproductively isolated from other such groups, will eventually develop their own unique mixture of mitochondrial genotypes (haplotypes).

The four species of salmonids in Tasmania [*Salmo salar*, *S. trutta*, *Oncorhynchus mykiss* (Smith and Stearley, 1989) and *Salvelinus fontinalis*] have been translocated from their original ranges in the northern hemisphere and all have been maintained in hatcheries at some stage in their recent history (MacCrimmon and Marshall, 1968; MacCrimmon and Campbell, 1969; MacCrimmon, 1971). With the recent stocking of Great Lake with Atlantic salmon, all species are found in the abundant and productive Tasmanian freshwater environment and are the basis of a renowned recreational fishery. Only brown trout populations, and to a lesser extent those of the rainbow and brook trout, are sustained by natural spawning. Two species, rainbow trout and Atlantic salmon, are cultured in fresh and sea water to supply local and international food markets.

The Tasmanian stock of Atlantic salmon originated from a naturally occurring spawning population in western Canada (River Phillip, Nova Scotia; Hortle, 1988). Three batches each of 100 000 eyed ova were taken in 1963–65 and shipped to a hatchery on mainland Australia. There are no detailed records of the survival of the imported stock on the mainland, but at one time the number of salmon in the hatchery was extremely low (D. Stocks, pers. comm., Gaden Trout Hatchery, P.O. Box 11, Jindabyne, New South Wales, Australia). Large numbers of salmon ova were quarantined into Tasmania in 1984. Rainbow trout were successfully introduced to Tasmania in 1904 from New Zealand. Subsequent importations continued from New Zealand until 1964 (G. French, pers. comm., Tasmanian Inland Fisheries Commission, 127 Davey Street, Hobart, Australia). New Zealand waters first received trout in 1883 from a steelhead stock collected from Russian River, California (MacCrimmon, 1971). Tasmanian brown trout were originally derived from a few hundred individuals imported as ova from The Wey and River Itchen (southern England) in 1864 (Walker, 1988). Additional shipments were made up until 1933, including the introduction of sea-running trout from Scotland (Clements, 1988). Brook trout in Tasmania are descended from a brood stock of 85 females and 102 males derived from 50 000 ova from the Cobequid Fish Culture Station at Collingwood, Nova Scotia, Canada in 1962 (Clements, 1988).

The general aims of this paper are two-fold; firstly, to use the unique properties of mtDNA to investigate the geographic origins of the four species of salmonids currently found in Tasmania and, secondly, to speculate on the

effect that domestication and translocation to Tasmania has had on the mtDNA genetics of the four species.

MATERIALS AND METHODS

Tasmanian salmonid species were sampled for genetic analyses in 1987 and 1988. Atlantic salmon ($n=30$) were sampled from two sea-farms and from the Salmon Ponds hatchery, New Norfolk (2). Rainbow trout were obtained from five hatchery strains which included triploids (8), all-females (8), steelheads (9) albinos (9) and normal stock (41). Brook trout were sampled from the Salmon Ponds hatchery (10) and from Clarence Lagoon (8). Brown trout were obtained from the River Derwent at Boyer (19) and from the Lawrence Canal (8).

Eight restriction endonucleases (*Bam*HI, *Bgl*I, *Bgl*II, *Hin*dIII, *Pst*I, *Pvu*II, *Sma*I and *Xba*I) were chosen to survey the mitochondrial genomes on the basis that these enzymes would allow the most detailed comparisons to previously published work. The position and number of restriction sites recognized by the eight enzymes was determined using conventional techniques (Chapman and Powers, 1984; Ovenden et al., 1988, 1989). The majority of pairwise comparisons between the results of this and other studies were made with data from the same six, seven or eight enzymes; however, comparisons were made where only two (Hovey et al., 1989), three (Quattro et al., 1990) or four (Davidson et al., 1989a) enzymes were shared (Table 1).

RESULTS

Atlantic salmon

The 32 Tasmanian Atlantic salmon mitochondrial genomes analysed in this study were identical, and designated haplotype A. The mtDNA of 41 Scandinavian salmon analysed by Gyllensten and Wilson (1987; Sweden) and Palva et al. (1989; Finland) also conformed to the restriction site pattern of haplotype A. However, the mitochondrial genomes of 15 salmon from Canada (Birt et al., 1986) and a further 40 from England (Hovey et al., 1989) had an alternative haplotype (B). Haplotypes A and B differed by the presence or absence of both a *Bgl*I and a *Bgl*II site (Fig. 1).

Rainbow trout

There was no difference in the mitochondrial haplotypes of the 75 Tasmanian rainbow trout sampled from five separate strains. Four restriction sites (*Pst*I, *Sma*I and two *Xba*I sites) differentiated the 75 Tasmanian haplotypes (haplotype C) from those studied by Thomas et al. (1986, haplotype B). The mtDNA of trout studied by Berg and Ferris (1984, haplotype A) differed by two additional restriction sites (*Bam*HI, *Pst*I). Two restriction sites (*Bgl*I

TABLE 1

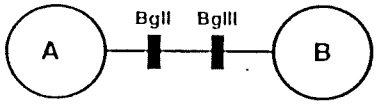
The sample size (n) and geographical origin of salmonid species whose mitochondrial genomes were compared in this study. The restriction enzymes common to this study and each previous study are indicated

Reference	n	Origin	Restriction enzyme							
			*Bam*HI	*Bgl*I	*Bgl*II	*Hin*dIII	*Pst*I	*Pvu*II	*Sma*I	*Xba*I
Atlantic salmon (*Salmo salar*)										
This study	32	River Phillip, Nova Scotia	•	•	•	•	•	•	•	•
Birt et al. (1986)	7	Bishops Falls, Exploits River, Canada	•	•	•	•	•	•		•
	8	Five Mile Pond East, Avalon Peninsula, Newfoundland	•	•	•	•	•	•		•
Gyllensten and Wilson (1987)	4	North Sea drainages, western Sweden	•	•	•	•	•	•	•	•
	5	Lule River, Baltic Sea	•	•	•	•	•	•	•	•
Davidson et al. (1989a)	1	Newfoundland	•			•	•			•
Hovey et al. (1989)	40	River Itchen, southern England*		•		•				
Palva et al. (1989)	23	Pielisjoki River, Finland	•		•	•	•	•		•
	9	Neva River, Russia	•		•	•	•	•		•
Rainbow trout (*Oncorhynchus mykiss*)										
This study	75	Russian River, California	•	•	•	•	•	•	•	•
Berg and Ferris (1984)	1	Unknown**	•	•	•	•	•	•		•
Thomas et al. (1986)	1	McCloud River, northern California	•	•	•	•	•		•	•
Gyllensten and Wilson (1987)	5	California	•	•	•	•	•	•	•	•
	5	Arlee Lake, California	•	•	•	•	•	•	•	•
	4	Eagle Lake, California	•	•	•	•	•	•	•	•
Brown trout (*Salmo trutta*)										
This study	27	Southern England and Scotland	•	•	•	•	•	•	•	•
Berg and Ferris (1984)	1	Unknown	•	•	•	•	•	•		•
Gyllensten and Wilson (1987)	5	Gullspång, Sweden	•	•	•	•	•	•	•	•
	4	Åvaån, Sweden	•	•	•	•	•	•	•	•
Brook trout (*Salvelinus fontinalis*)										
This study	18	Collingwood, Nova Scotia	•	•	•	•	•	•	•	•
Berg and Ferris (1984)	1	Unknown	•	•	•	•	•	•		•
Gyllensten and Wilson (1987)	3	Wings Pond, Newfoundland*	•		•	•	•	•	•	•
Grewe et al. (1990)	2	Lake Nipigon, Ontario*	•	•	•	•	•	•	•	•
	2	Dickson Lake, Ontario*	•	•	•	•	•	•	•	•
	12	Temiskaming District, Ontario	•	•	•	•	•	•	•	•
Quattro et al. (1990)	143	Western Maryland, US*				•	•			•

*Denotes wild caught specimens. The remainder are from hatcheries.

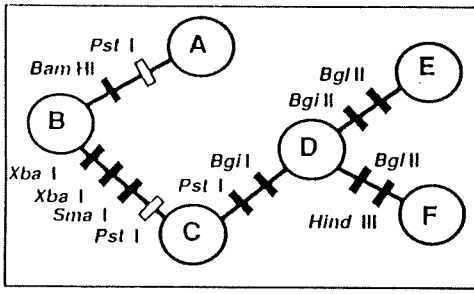
**Six fish were analysed in this study, but they effectively represent genetic variation from only one, as the fish were from the same maternal family.

Atlantic Salmon



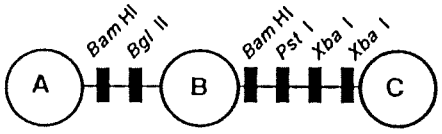
Origin	Reference
A Sweden	Gyllensten and Wilson (1987)
Finland	Palva *et al.* (1989)
River Phillip, Nova Scotia	This study
B Newfoundland	Birt *et al.* (1986), Davidson *et al.* (1989)
England	Hovey *et al.* (1989)

Rainbow Trout



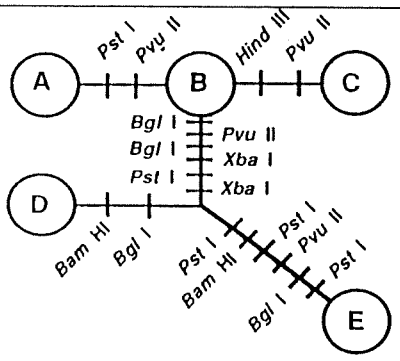
Origin	Reference
A California	Berg and Ferris (1984)
B Northern California	Thomas *et al.* (1986)
C Russian River, California	This study
D Eagle Lake, California	Gyllensten and Wilson (1987)
E California	Gyllensten and Wilson (1987)
F Arlee Lake, California	Gyllensten and Wilson (1987)

Brown Trout



Origin	Reference
A Sweden	Gyllensten and Wilson (1987)
B England	This study
C California hatchery	Berg and Ferris (1984)

Brook Trout



Origin	Reference
A Hatchery, Nova Scotia	This study
Western Maryland	Quattro *et al.* (1990)
B Hatchery, Nova Scotia	This study
Western Maryland	Quattro *et al.* (1990)
C Wings Pond, Newfoundland	Gyllensten and Wilson (1987)
D Hatchery, Ontario	Grewe *et al.* (1990)
Wild, Ontario	Grewe *et al.* (1990)
E Hatchery, California	Berg and Ferris (1984)

Fig. 1. The relationship between mitochondrial DNA haplotypes of four salmonid species based on their possession, or otherwise, of restriction endonuclease recognition sites. The restriction endonuclease site, whose state varies between haplotypes, is indicated by a cross-wise bar. The open bar shows that the same restriction site has been gained or lost in more than one lineage. Restriction fragment sizes for each haplotype can be obtained from the senior author.

and *Pst*I) separated this group of haplotypes (A, B and C) from the three trout haplotypes studied by Gyllensten and Wilson (1987; D, E and F). These haplotypes differed from each other by two or four restriction sites (*Bgl*II and *Hin*dIII, Fig. 1).

Brown trout

As with Tasmanian samples of Atlantic salmon and rainbow trout genomes, the 27 Tasmanian brown trout mitochondrial genomes were identical when examined with the eight restriction enzymes. The mtDNA of Tasmanian brown trout (haplotype B) differed from that of Swedish trout (haplotype A; Gyllensten and Wilson, 1987) by two restriction sites recognized by *Bam*HI and *Bgl*I. Four restriction sites differed between the mtDNA of the Tasmanian trout and those of an unknown origin studied by Berg and Ferris (1984, haplotype C, Table 1, Fig. 1).

Brook trout

Two brook trout haplotypes were identified among the 18 Tasmanian individuals included in this study. Using data from three restriction enzymes only (*Hin*dIII, *Pst*I and *Xba*I), 143 brook trout from West Maryland, USA (Quattro et al., 1990) were identical to these two Tasmanian haplotypes. Haplotype A (Tasmania, 17/18 fish; Quattro et al., 1990, 112/143) differed from haplotype B (Tasmania, 1/18 fish; Quattro et al., 1990, 31/143) by two restriction sites (*Pst*I and *Pvu*II). Brook trout from Wings Pond, Newfoundland (haplotype C, Gyllensten and Wilson, 1987) differed from haplotype B by a further two restriction sites. Haplotypes D (Ontario; Grewe et al., 1990) and E (unknown origin; Berg and Ferris, 1984) differed from haplotypes A, B and C by six restriction sites. Haplotype D differed by a further two restriction sites and haplotype E by six restriction site changes (Table 1, Fig. 1).

Restriction sites cleaved by the enzyme *Pst*I identified a polymorphism in the Tasmanian brook trout. Seventeen out of the 18 genomes sampled had two restriction sites which produced two fragments, approximately 12 900 and 3800 nucleotide pairs in length. The remaining genome possessed only one *Pst*I site. This polymorphism was identical to the one reported by Quattro et al. (1990). Thirty-one of their 143 brook trout genomes had two *Pst*I sites, while the remainder (112) possessed only one *Pst*I site.

The restriction sites of *Pvu*II also identified a polymorphism in Tasmanian brook trout. Seventeen of 18 genomes had two restriction sites, about 8900 or 7900 nucleotide pairs apart. The remaining genome had an extra *Pvu*II site occurring within the 7900 nucleotide fragment, approximately 1500 nucleotides from one end. This variant genome was the same one that possessed the variant *Pst*I restriction site pattern. Quattro et al. (1990) did not digest their samples with *Pvu*II.

DISCUSSION

Allele frequency data from 38 electrophoretic loci identified a major division between Atlantic salmon derived from mainland Canada and Europe (Stahl, 1987). The availability of mtDNA data led Davidson et al. (1989b) to suggest that a polymorphism in *Bgl*II restriction sites may coincide with the trans-Atlantic division of the species. This hypothesis was supported in a subsequent analysis of the mtDNA of 17 salmon from North America and Canada which had a single *Bgl*II site, while the genomes of 30 salmon from Europe had two *Bgl*II sites (Bermingham et al., 1991). However, the results of Birt et al. (1991) do not conform to this pattern. Birt et al. (1991) reported both forms of the polymorphism in genomes of 71 salmon from Gambo Pond in Newfoundland, Canada. Using four additional restriction enzymes (*Bst*EII, *Cla*I, *Dra*I and *Ava*II), Bermingham et al. (1991) were able to separate salmon with a western or eastern Atlantic origin. However, as they acknowledge, even these genetic markers are not definitive. In view of the lack of distinction between the mitochondrial genomes of Atlantic salmon derived from either mainland Canada or Europe based on particular restriction site polymorphisms, it is not possible to use our data on the mtDNA of Tasmanian Atlantic salmon to test their supposed geographical origin (River Phillip, Nova Scotia; Hortle, 1988).

Atlantic salmon are renowned for their ability to seek out their natal freshwater streams for mating and spawning (Scott and Crossman, 1973). In fish species with this kind of life history strategy, intraspecific mtDNA nucleotide sequence diversity would be expected to be high as the resultant reproductive isolation would promote mtDNA lineage sorting (Avise et al., 1984). For example, in the chinook salmon (*Oncorhynchus tshawytscha*), which has a similar life history to the Atlantic salmon, six mitochondrial haplotypes were identified among 76 fish (Wilson et al., 1987). However, we report only two Atlantic salmon haplotypes amongst six studies of a total of 129 specimens. The addition of three haplotypes amongst the 47 salmon analysed by Bermingham et al. (1991) with a suite of 20 restriction enzymes does not alter the observation that the species is low in mtDNA restriction site polymorphism.

The relatively large amount of mtDNA diversity between the Tasmanian and North American stocks of rainbow trout compared in this study (at least two to four restriction sites, Fig. 1) may reflect a correspondingly large amount of diversity between the wild populations from which they were derived. In the numerous years since the domestication of hatchery stocks of rainbow trout, mtDNA diversity in wild populations may have been reduced from the combined effects of population decline followed by indiscriminant re-stocking. If this is the case, the genetic diversity represented in worldwide hatchery

populations of rainbow trout is a valuable resource which should be carefully conserved for the future.

The apparent lack of mtDNA diversity in Tasmanian rainbow trout stocks may be due to the transitory maintenance of the strain in hatcheries during the last 100 years or more. MtDNA lineage diversity is lost when the offspring of many maternal lineages are repeatedly cultured in a mixed stock and are the source of randomly chosen and less numerous replacement brood stock. Differential survival of maternal lineages in this type of culture system, over many generations, can cause the gradual, and unintentional, extinction of mtDNA lineages. Despite this effect, however, some Finnish hatcheries have managed to retain mtDNA sequence diversity in their domesticated rainbow trout. On the basis of only three restriction enzmyes (*Bgl*II, *Eco*RI and *Hin*dIII), Palva and Palva (1987) reported four mtDNA haplotypes amongst 38 specimens from three of four Finnish hatchery strains. Five of the fish from a strain originating from non-anadromous Kamloops stock from Canada possessed two haplotypes. Twelve fish from a Danish Californian-derived strain possessed three haplotypes and 13 fish from an American hybrid strain possessed two haplotypes.

Tasmanian populations of brown trout were originally derived from a few hundred individuals which were imported from England in 1864 (Walker, 1988). Additional shipments were made up until 1933, including the introduction of sea-running trout from Scotland (Clements, 1988). As large numbers of mtDNA haplotypes appear to be a consistent feature of most naturally occurring, vertebrate populations, especially in freshwater fish (Bermingham and Avise, 1986; Ovenden and White, 1990), the haplotype diversity of brown trout in the British Isles probably was at that time, and is still today (Hovey et al., 1989), relatively high. Consequently, it is likely that each importation of brown trout to Tasmania may have introduced numerous, novel haplotypes, especially if each consignment had a different geographical origin. Today, brown trout are found in virtually every lacustrine and riverine habitat in Tasmania (French, 1987), suggesting that acclimation efforts were generally successful. Clements (1988) noted that the 1864 introduction of ova produced vigourous wild populations in varying locations throughout Tasmania and speculated that subsequent introductions made little difference to the success of the species. If post-1864 introductions of brown trout to Tasmania did not give rise to self-sustaining populations, then the sea-running brown trout populations which are found in many Tasmanian coastal rivers may not have a sea-running, Scottish ancestry but may be derived from the original non-marine population in southern England. The single haplotype possessed by all of the Tasmanian individuals sampled, from inland (8) and coastal (19) waterways, suggests that the present-day brown trout stocks in Tasmania are the descendants of a single introduction. To confirm this, it would be necessary to analyse the mtDNA of brown trout from a more extensive

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APPENDIX E

Smolenski, A.J., Ovenden, J.R. & White, R.W.G. 1993. Evidence of stock separation in southern hemisphere orange roughy (*Hoplostethus atlanticus*, Trachichthyidae) from restriction enzyme analysis of mitochondrial DNA. *Marine Biology* **116**, 219-230. (Appendix E)

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Evidence of stock separation in southern hemisphere orange roughy (*Hoplostethus atlanticus*, Trachichthyidae) from restriction-enzyme analysis of mitochondrial DNA

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Abstract. Restriction enzyme analysis of mitochondrial DNA (mtDNA) was used to test for genetic homogeneity of orange roughy (*Hoplostethus atlanticus*) in the southern hemisphere. Two hundred and eighty-six orange roughy specimens were collected from seven general localities: the Great Australian Bight; South Australia (off southeastern Kangaroo Island); the west coast of Tasmania; the east coast of Tasmania; New South Wales; New Zealand and South Africa. Mitochondrial DNA was extracted from developing ovary tissue and analysed with 10 six-base enzymes and 3 four-base enzymes. Both forms of analysis revealed a low level of genetic diversity in this species. The six-base enzyme study found no evidence of reproductively isolated populations of orange roughy in southeastern Australian waters. However, an analysis of 107 fish with 3 four-base enzymes identified at least partial genetic separation of the New South Wales (NSW) sample of orange roughy from South Australian (off southeastern Kangaroo Island) and Tasmanian samples. This finding supports biological evidence for the presence of a distinct subpopulation of orange roughy in NSW waters. The four-base study also provided evidence of the presence of genetically distinct samples of orange roughy occurring in the same localities off southeastern Kangaroo Island from consecutive years. Additional sampling and the use of a greater number of four-base enzymes may be needed to determine if any genetic structuring exists among orange roughy south of New South Wales.

Introduction

The orange roughy (*Hoplostethus atlanticus*, Trachichthyidae) is a commercially important demersal marine teleost which, in recent years, has been heavily exploited in southeastern Australian waters. Logbook records have shown that from 1989 to 1990, ~ 34 000 t of orange roughy were taken from Tasmanian waters at an estimated return of 50 million Australian dollars (Lyle et al. 1990). Large aggregations of orange roughy have been located off northeastern and southwestern Tasmania, in western Bass Strait off Portland, southeast of Kangaroo Island, and in the Great Australian Bight. It is now known that at least two of these aggregations, one off northeastern Tasmania and the other off Port Lincoln in the Great Australian Bight, were spawning aggregations (Lyle et al. 1989, 1990, Newton et al. 1990).

The orange roughy is distributed worldwide. It occurs at depths between 750 and 1150 m and probably extends down to 1350 m (Robertson et al. 1984). Fish grow to 45 cm standard length, with females tending to be larger than males (Lyle et al. 1989). Orange roughy is a winter spawner with a low relative fecundity (Pankhurst and Conroy 1987), and exhibits some variation in the timing of spawning. Bell (1989) demonstrated that fish sampled from New South Wales waters in 1988 ceased spawning by mid-June, whereas fish sampled from South Australian and Tasmanian waters spawned mainly in the second half of July. Newton et al. (1990) observed that orange roughy in the Great Australian Bight completed spawning by the third week of August.

Recent age-estimate studies indicate that orange roughy may be particularly long-lived. Radiometric analysis carried out by Fenton et al. (1991) indicated that individuals from 38 to 40 cm in length may be between 77 and 149 yr of age, with age at maturity (32 cm standard length) being ~32 yr. These results give a higher estimate of the maximum age of orange roughy than the study of otolith rings carried out by Mace et al. (1990), who estimated the average age at maturity as ~20 yr with the maximum age possibly exceeding 50 yr. Information on the larval biology of orange roughy remains incomplete.

In an allozyme study of orange roughy which identified 22 scoreable loci, Smith (1986) found very little genetic differentiation between samples collected from the Atlantic Ocean and the Tasman–Pacific region, areas separated by a distance of ~21 000 km. Because of the low level of nuclear gene heterozygosity found in orange

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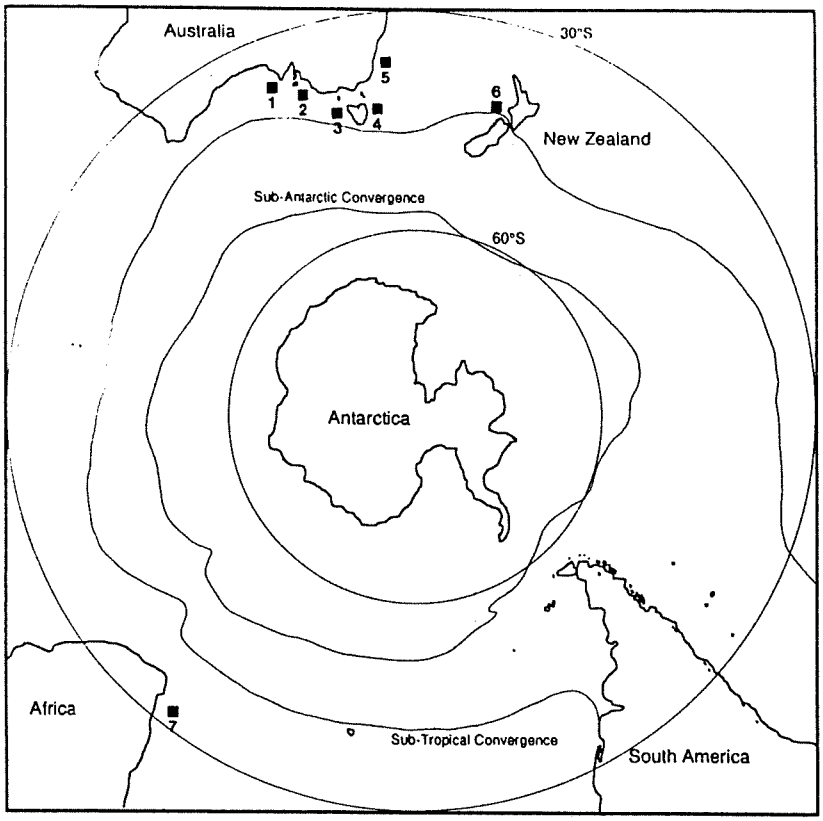

Fig. 1. Main sampling areas of orange roughy, *Hoplostethus atlanticus*. 1: Great Australian Bight, 2: Kangaroo Island (referred to as "S Aust" in Tables 1 to 7), 3: western Tasmania, 4: eastern Tasmania, 5: New South Wales, 6: New Zealand, 7: South Africa

roughy, Smith could not reject the null hypothesis that the fish were drawn from a single interbreeding unit. However, in an allozyme study of Australian and New Zealand samples of orange roughy, Black and Dixon (1989) identified three subpopulations of orange roughy: New Zealand, eastern Australia and Tasmania, and South Australia, on the basis of discontinuities in the allele frequencies of nine polymorphic loci. Elliott and Ward (1993), in a study which substantially overlapped that of Black and Dixon, screened 11 polymorphic loci in orange roughy collected from a wide range of southern Australian localities. They failed to detect any evidence of stock separation.

Three other studies have provided evidence of stock separation in orange roughy. On the basis of parasite faunas, Lester et al. (1988), suggested that there are five Australian and three New Zealand stocks of orange roughy. A study of the mitochondrial DNA (mtDNA) sequence diversities of two Tasmanian samples of orange roughy found preliminary evidence of the subdivision of rare haplotypes between the east and west coast of Tasmania (Ovenden et al. 1989). A recent study of the otolith microchemistry of orange roughy has suggested the presence of three stocks of orange roughy in southeastern Australian waters (Edmonds et al. 1991).

The present paper examines the genetic structure of orange roughy samples using restriction enzyme analysis of mtDNA. Mitochondrial DNA in animal cells is a small, covalently closed, circular molecule, ~16 to 20 000 base pairs long. Animal mtDNA undergoes relatively simple forms of evolutionary change and is clonaly inherited through the female lineage, therefore the genome is uncomplicated by recombination (Avise et al. 1987a, Birky et al. 1989). The relative rate of mtDNA evolution is ~5 to 10 times faster than single-copy nuclear DNA, or ~0.02 substitutions per base pair per million years (Brown et al. 1979). Restriction-enzyme analysis of mtDNA estimates the relative amount of nucleotide-sequence diversity in the mitochondrial genome and, by inference, the degree of genetic subdivision between animal groupings. The assumption is that once a barrier to gene flow has been established between individuals from the same population, different mutations in the mtDNA will arise and be perpetuated within each subpopulation. It is anticipated that the greater resolving power of mtDNA analysis, as opposed to other molecular techniques such as allozyme electrophoresis, may provide a better means of identifying the stock boundaries of orange roughy in southeastern Australian waters.

Materials and methods

Sampling

Two hundred and eighty-six mtDNA samples (mainly developing ovary tissue) were analysed from specimens of *Hoplostethus atlanticus* measuring 34.7±2.9 cm (mean±SD) and weighing 1246.9±597.7 g (mean±SD) collected at seven general locations. These were the Great Australian Bight, South Australia (off southeastern Kangaroo Island) the west and east coasts of Tasmania,

New South Wales, New Zealand and South Africa (Fig. 1). Samples were collected off the west and east coasts of Tasmania over a period of 3 yr (1987, 1988 and 1989), the South Australian samples were collected over a period of 2 yr (1988 and 1989). Details of the exact sampling localities and time of sampling can be obtained from the senior author.

Most of the samples were collected by staff at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Division of Fisheries and the Tasmanian Division of Sea Fisheries. The South African samples were collected by the Department of Environment Affairs, Marine Development, Rogge Bay, Capetown. The New Zealand samples were provided by the New Zealand Ministry of Agriculture and Fisheries in Wellington.

Orange roughy provided by the CSIRO Division of Fisheries were collected during a series of random stratified trawl surveys (Bulman et al. 1993) in the Kangaroo Island region of the Great Australian Bight and off the west and east coasts of Tasmania. The Tasmanian Division of Sea Fisheries also carried out a series of random stratified trawl surveys along the west and east coasts of Tasmania. Many of the orange roughy samples used in this study were collected from spawning aggregations, particularly during the random trawl surveys conducted by the Tasmanian Division of Sea Fisheries.

Restriction-enzyme analysis

The mtDNA of 283 individuals were screened with each of 10 six-base restriction enzymes according to the suppliers' specifications. The 49 orange roughy individuals analysed in a pilot study (Ovenden et al. 1989) were added to the present set of 283 individuals for the purpose of *G*-statistic (G_{st}) and group-variance analysis. The mtDNA extraction procedure, restriction enzyme reaction conditions, as well as end-labelling and electrophoresis methods, are described in Ovenden et al. (1989). A subset of 107 individuals, which were representative of each major locality, were chosen from those analysed with six-base enzymes (including three individuals not screened with six-base enzymes), and analysed with three enzymes recognizing sequences of four bases.

Radiolabelled mtDNA fragments produced by four-base restriction endonucleases were loaded onto a 3.5 or 5% polyacrylamide gel, 360 mm long and 0.25 mm thick, in Tris (0.089 *M*)-boric acid (0.089 *M*)-EDTA (0.025 *M*) buffer (pH 8.2). A size standard was provided by lambda DNA digested with *Bgl*I. Gels were run for ~1 h at 20 mA or until the bromophenol-blue tracking dye was more than 75% of the way into the gel. The gels were dried and autoradiographed for up to one week.

Mitochondrial genome mapping

The restriction sites recognized by analysis with six-base restriction enzymes were mapped upon the orange roughy mitochondrial genome using the double-digest technique (Sambrook et al. 1989). A lambda DNA size-standard generated by *Hin*dIII was included on each gel for fragment-size estimation. The fragment sizes were converted to number of base pairs mapped clockwise from one of two *Sac*II sites (see Fig. 2). The mapping of the orange roughy genome was aided by the use of a computer package written for this purpose (Ovenden et al. 1992b).

Data analysis

Unique fragment patterns identified by each of the ten restriction enzymes were assigned an upper-case letter and referred to as restriction morphs. The most common fragment pattern was assigned the letter "A". The loss or gain of a restriction site was determined by the loss or gain of restriction fragments of the appropriate size.

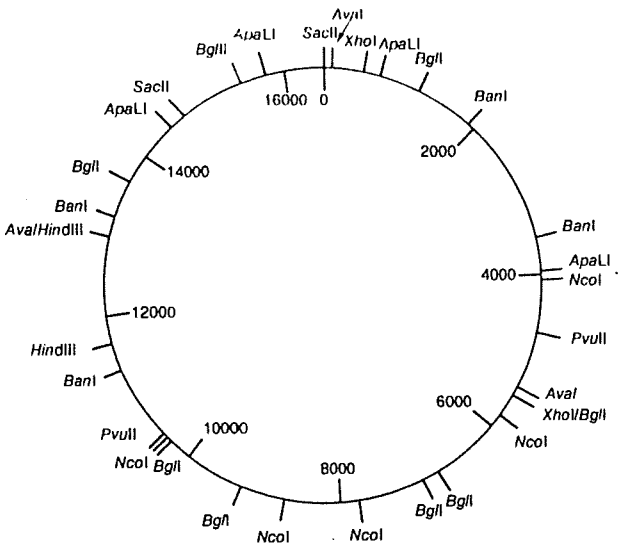

Fig. 2. *Hoplostethus atlanticus*. Mitochondrial genome (common haplotype AAAAAAAAAA); restriction sites mapped clockwise from one of two *Sac*II sites. Size scale for restriction sites (in base pairs) is inside circle

The site presence or absence data were entered onto a computer in a binary code form for analysis.

The four-base haplotypes were labelled alphabetically from A to Z and then from A' to Z'. It was difficult to determine four-base restriction site gains and/or losses between individuals due to the large number of sites. Therefore, fragment presence or absence data were arranged into a binary code form and entered onto a computer for analysis.

An estimate of the amount of interpopulational genetic subdivision of orange roughy samples was gained using G_{st} analysis. The *G*-statistic (G_{st}) is equal to the amount of variation in the whole data set that is due to the division of the population into a series of interbreeding populations. Eqs. (17) and (19) of Takahata and Palumbi (1985) were used to calculate the intrademe and interdeme identity probability using restriction-site presence or absence data from each population. The significance of the G_{st} analysis was evaluated by bootstrapping (Palumbi and Wilson 1990).

The G_{st} analysis of the six-base enzyme results used restriction-site data in binary form. However, for four-base data, G_{st} analysis was carried out on the presence or absence of restriction fragment data. The loss or gain of a restriction fragment is not an independent event, unlike the loss or gain of a restriction site. Therefore, the loss of a restriction site results in a new restriction fragment which replaces two previous restriction fragments and vice versa. However, Kessler and Avise (1984) argue that this redundancy of information applies only to fragments produced by single enzymes, one at a time. Furthermore, this information is not completely redundant, since a given site gain can result in the formation of any one of a number of new pairs of fragments, depending on the position of the site gain on the genome. The use of a large data set involving a large number of restriction fragments, as used in the present study, should overcome the problem of redundant information.

A χ^2-test was used to compare the observed and expected four-base restriction-morph frequencies. The significance of the χ^2-value obtained was tested using the Monte-Carlo method (Roff and Bentzen 1989) and was compared with the χ^2-values obtained from 1000 randomizations of each data set. The six-base enzyme haplotype data were highly skewed and it was deemed inappropriate to use the χ^2-test.

For the six-base enzyme data, the maximum likelihood method of Nei and Tajima (1983) was used to calculate the number of base substitutions per base pair (δ between pairs of individual fish and

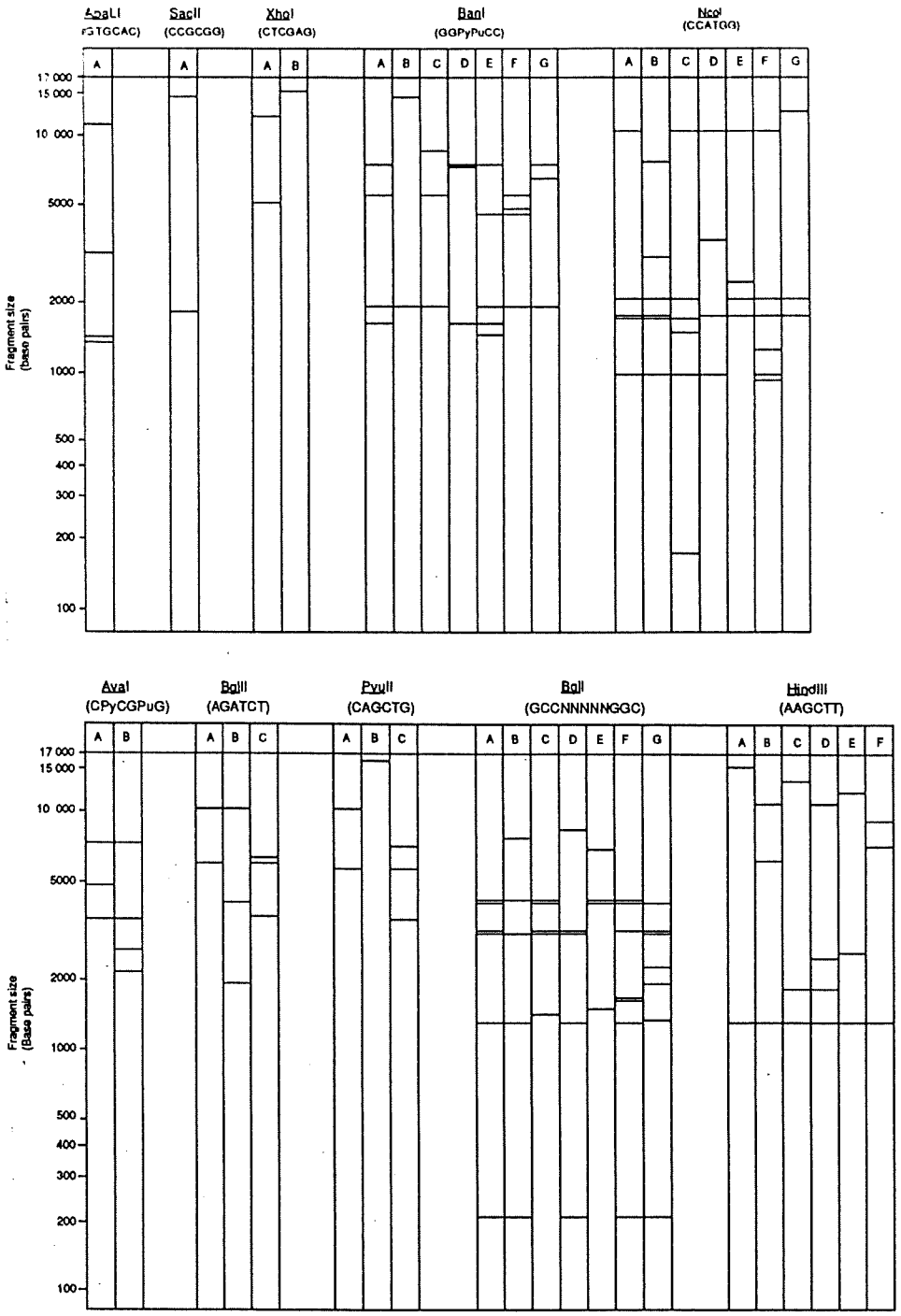

Fig. 3. *Hoplostethus atlanticus.* Fragment patterns of mitochondrial haplotypes identified by 10 six-base restriction enzymes. Recognition sequences of restriction enzymes are in parentheses

between pairs of composite haplotypes. The δ value and the standard deviation of δ ($V\delta$) were calculated using Eqs. (25) and (11), respectively, in Nei and Tajima. An estimate of δ was calculated from the presence or absence of four-base restriction-fragment data using Eq. (20) in Nei and Li (1979), and its standard deviation was calculated from Eq. (11) in Nei and Tajima (1983).

An estimate of the mean net δ between populations was gained using Eq. (16) of Nei and Jin (1989). The group variance of δ was calculated using Eq. (3) of Nei and Jin. A Student's t-test was used to determine whether the net diversity estimates were significantly different from zero. However, it should be noted that the pairwise comparison of net diversity estimates between localities is not statistically independent (see Table 7). By calculating the group variance it was possible to test statistically whether Population A was significantly different from Population B using the null hypothesis that the net $\delta = 0$. Furthermore, it tested if Population A was as similar to Population B as A was to Population C, with the null hypothesis being that $\delta AB = \delta AC$. Although methods of data analysis such as G_{st} and χ^2 are useful in identifying population subdivision, an analysis of the group variance complements these methods by giving a

Table 1. *Hoplostethus atlanticus*. Locality and number of orange roughy identified with six-base enzyme analysis. GAB 1988: Great Australian Bight 1988; S Aust 1988, S Aust 1989: South Australia (off southeastern Kangaroo Island) 1988, 1989, respectively; W Tas 1987, W Tas 1988, W Tas 1989: Western Tasmania 1987, 1988, 1989, respectively; E Tas 1987, E Tas 1988, E Tas 1989: Eastern Tasmania 1987, 1988, 1989, respectively; NSW 1988: New South Wales 1988; NZ 1988: New Zealand 1988; S Africa 1989: South Africa 1989. The order of the ten restriction enzymes in the composite haplotypes is: *Apa*LI, *Ava*I, *Ban*I, *Bgl*I, *Bgl*II, *Hin*dIII, *Nco*I, *Pvu*II, *Sac*II and *Xho*I

Haplotype	GAB 1988	S Aust 1988	S Aust 1989	W Tas 1987[a]	W Tas 1988	W Tas 1989	E Tas 1987[a]	E Tas 1988	E Tas 1989	NSW 1988	NZ 1988	S Africa 1989	Total
AAAAAAAAAA	7	47	30	17	26	27	19	40	21	5	6	18	263
AAAAAAAAAB		1				1			1				3
AAAAAAABAA			1				1	2		1		1	6
AAAAAAACAA					1								1
AAAAAABAAA							1					2	3
AAAAAACAAA		1											1
AAAAAADAAA								1					1
AAAAAAEAAA			2										2
AAAAAAGAAB												1	1
AAAAABAAAA				1				1					2
AAAAACAAAA		1		2								1	4
AAAAADAAAA			1										1
AAAAAEAAAA												1	1
AAAABABAAA							1						1
AAAACAAAAA		2		1						1		1	5
AAABAAAAAA							1						1
AAACAAAAAA		1	1	1									3
AAACAAFAAA											1		1
AAADAAAAAA								1					1
AAAEAAAAAA								1					1
AAAFAAAAAA		1	1										2
AAAGAAAAAA					1								1
AABAAAAAAA				1	1								2
AABACAAAAA		1											1
AACAAAAAAA		3		3		1				1		1	9
AADAAAAAAA		1				3		1	1		2		8
AAEAACAAAA											1		1
AAFAAAAAAA		1											1
AAGAAAAAAA												1	1
AAGAAAABAA												2	2
ABAAAAAAAA								2					2
Total	7	60	36	26	29	32	23	49	23	8	10	29	332

[a] Data from Ovenden et al. (1989)

far more detailed picture of population subdivision. The use of the group-variance method has been discussed by Ovenden et al. (1992a).

Results

Six-base enzyme survey

The size of the mitochondrial genome of *Hoplostethus atlanticus* was estimated by averaging the sum of all the fragments for each enzyme, giving a value of 16 481 ± 364 base-pairs (mean ± standard deviation).

The survey of 283 orange roughy mitochondrial genomes with 10 six-base restriction enzymes recognized 43 restriction sites. Two of the ten restriction enzymes used to screen the mitochondrial genome of orange roughy, *Apa*LI and *Sac*II, revealed no restriction-site variation. Between 1 and 6 rare fragment patterns were identified by each of the remaining 8 restriction enzymes. The enzymes *Ava*I and *Xho*I each identified 1 unique morph in addition to the common morph, whilst *Bgl*II and *Pvu*II each produced 2 rare morphs in addition to the common morph. The enzyme *Hin*dIII yielded 5 unique morphs, *Ban*I, *Bgl*I and *Nco*I each produced 6 unique mitochondrial morphs. The fragment patterns for all 10 six-base enzymes are illustrated in Fig. 3.

Thirty rare composite haplotypes (including haplotypes from Ovenden et al. 1989) were identified during the six-base enzyme analysis. A "rare" haplotype is defined as any haplotype other than the common one (AAAAAAAAAA). Each rare haplotype was represented by 1 to 9 individuals. The rare haplotypes represented by more than two individuals (AAAAAAAAAB, AAAAACAAAA, AAACAAAAAA, AADAAAAAAA, AACAAAAAAA, AAAACAAAAA, AAAAAABAAA and AAAAAAABAA) tended to be geographically widespread; no rare haplotype was represented by more than three individuals at any one locality (Table 1).

The bootstrapped G_{st} value calculated from restriction-site presence or absence data for the seven localities where orange roughy were collected was 35%. However, this figure does not indicate population subdivision, as it

lies within a range of 30 and 38% calculated from 1000 bootstrapped G_{st} estimates of the original data set.

The analysis of the six-base nucleotide-sequence diversity data was divided into two parts. Firstly, all fish collected from southeastern Australian waters (all localities except New Zealand and South Africa) were analysed together to test the null hypothesis that there is no reproductive isolation amongst the five southeastern Australian samples. The South African and New Zealand samples were then analysed separately, along with a combined sample of all of the fish collected from the west coast of Tasmania in 1988 and 1989, which were chosen to be representative of southeastern Australian fish.

The mtDNA diversity for the 244 individuals collected from southeastern Australian waters was $0.1262 \pm 0.0449\%$ (mean $\pm$ SE). The results of the intrapopulational mtDNA diversity analysis are summarised in Table 2.

Forty-five interpopulational comparisons of nucleotide diversity between the mtDNA of orange roughy individuals were carried out from pairs of localities in southeastern Australian waters. All the interpopulational comparisons produced net nucleotide diversities approximating zero, and in 43 of the 45 comparisons the nucleotide diversities were less than or equal to their corresponding standard error. The low net interpopulational sequence diversities for the southeastern Australian samples suggests an absence of geographical structuring of orange roughy stocks based on the six-base haplotype analysis.

A comparison of orange roughy samples collected from western Tasmanian, New Zealand and South African waters yielded a total mtDNA diversity of $0.1939 \pm 0.0876\%$, ($N = 100$). The results of the intrapopulational analysis are summarised in Table 2. Pairwise comparisons of these localities yielded net mtDNA diversities which were smaller than their corresponding standard errors, indicating no significant difference in net mtDNA sequence diversity between the three localities.

Four-base enzyme survey

The survey of 107 orange roughy individuals from seven sample sets (South Australia 1988, $N = 20$; 1989, $N = 19$; western Tasmania 1988, $N = 20$; eastern Tasmania 1988, $N = 13$; New South Wales, $N = 7$; New Zealand, $N = 9$ and South Africa, $N = 19$) with three four-base enzymes produced 130 different fragments and identified 104 unique composite haplotypes. Therefore, the vast majority of the composite haplotypes were represented by just one individual. Individually, the three restriction enzymes *Mbo*I, *Msp*I and *Taq*I produced 45, 46 and 33 unique morphs, respectively.

The bootstrapped G_{st} value calculated from restriction-fragment presence or absence data for the seven sample sets was 26%. This value is well outside the range of 18 to 21% calculated from 1000 randomized G_{st} estimates. This result suggests population subdivision.

A χ^2-test using Monte-Carlo simulation was used to compare the observed and expected frequencies for each enzyme-specific morph that occurred more than once (Tables 3, 4, 5). Restriction morphs that occurred only once were excluded in order to avoid inflated significance values.

Each χ^2-test carried out to test the homogeneity of the enzyme specific morphs for all the localities returned a significant result (*Mbo*I, $P \leq 0.001$; *Msp*I, $P \leq 0.001$; and *Taq*1, $P \leq 0.001$).

Table 2. *Hoplostethus atlanticus*. Mean intrapopulational mtDNA diversity estimates and standard errors (%) for 12 orange roughy samples analysed with six-base enzymes. Abbreviations as in Table 1

Collection locality	Diversity	(SE)	*N*
GAB 1988	0	(0)	7
S Aust 1988	0.1416	(0.0810)	60
S Aust 1989	0.1035	(0.0654)	36
W Tas 1987[a]	0.2795	(0.1335)	26
W Tas 1988	0.0552	(0.0452)	29
W Tas 1989	0.0988	(0.0732)	32
E Tas 1987[a]	0.1661	(0.0810)	23
E Tas 1988	0.1214	(0.0686)	49
E Tas 1989	0.0466	(0.0397)	23
NSW 1988	0.2028	(0.1471)	8
NZ 1988	0.4243	(0.1659)	10
S Africa 1989	0.3495	(0.1533)	29

[a] Data from Ovenden et al. (1989)

Table 3. *Hoplostethus atlanticus*. Geographical locality and number of orange roughy *Mbo*I haplotypes that occurred more than once. Abbreviations as in Table 1

Locality	Haplotype									
	A	B	C	D	E	F	G	H	I	J
S Aust 1988	5	0	0	0	0	0	0	0	0	0
S Aust 1989	5	0	0	0	2	0	2	2	2	0
W Tas 1988	3	3	3	0	0	0	0	0	0	2
E Tas 1988	7	0	0	0	0	2	0	0	0	0
NSW 1988	0	2	0	0	0	0	0	0	0	0
NZ 1988	0	2	3	2	0	0	0	0	0	0
S Africa 1989	0	11	2	0	0	0	0	0	0	0

Table 4. *Hoplostethus atlanticus*. Geographical locality and number of orange roughy *Msp*I haplotypes that occurred more than once. Abbreviations as in Table 1

Locality	Haplotype				
	A	B	C	D	E
S Aust 1988	4	3	0	0	0
S Aust 1989	2	0	2	3	0
W Tas 1988	3	0	10	0	0
E Tas 1988	4	0	0	4	0
NSW 1988	0	0	0	0	2
NZ 1988	7	0	0	0	0
S Africa 1989	10	8	0	0	0

Table 5. *Hoplostethus atlanticus*. Geographical locality and number of orange roughy *Taq*I haplotypes that occurred more than once. Abbreviations as in Table 1

Locality	Haplotype				
	A	B	C	D	E
S Aust 1988	1	7	4	0	0
S Aust 1989	11	0	0	0	0
W Tas 1988	2	0	2	7	3
E Tas 1988	5	2	0	0	0
NSW 1988	4	0	2	0	0
NZ 1988	4	0	3	0	0
S Africa 1989	12	0	0	0	0

Table 6. *Hoplostethus atlanticus*. Mean intrapopulational mtDNA diversity estimates and standard errors (%) for seven orange roughy populations analysed with four-base enzymes. Abbreviations as in Table 1

Collection locality	Diversity	(SE)	*N*
S Aust 1988	0.5714	(0.1532)	20
S Aust 1989	0.6745	(0.1712)	19
W Tas 1988	0.6682	(0.1907)	20
E Tas 1988	0.6229	(0.1740)	13
NSW 1988	0.5184	(0.2008)	7
NZ 1988	0.3628	(0.1639)	9
S Africa 1989	0.2974	(0.1346)	19

Table 7. *Hoplostethus atlanticus*. Mean interpopulational mtDNA diversity estimates and standard errors (%) for orange roughy genomes analysed with four-base enzymes. Underlined values denote possible significant differences in mtDNA diversity. Abbreviations as in Table 1

Collection locality		Diversity	(SE)
E Tas 1988	vs NSW 1988	0.1208	(0.0319)
E Tas 1988	vs S Africa 1989	0.0859	(0.0710)
E Tas 1988	vs S Aust 1988	0.0148	(0.0084)
E Tas 1988	vs S Aust 1989	0.0235	(0.0083)
E Tas 1988	vs W Tas 1988	0.1875	(0.1075)
E Tas 1988	vs NZ 1988	0.0732	(0.0269)
NSW 1988	vs S Africa 1989	0.0699	(0.0313)
NSW 1988	vs S Aust 1988	0.1743	(0.0273)
NSW 1988	vs S Aust 1989	0.1429	(0.0330)
NSW 1988	vs W Tas 1988	0.2792	(0.1204)
NSW 1988	vs NZ 1988	0.0629	(0.0298)
S Africa 1989	vs S Aust 1988	0.1089	(0.0639)
S Africa 1989	vs S Aust 1989	0.1230	(0.0765)
S Africa 1989	vs W Tas 1988	0.2158	(0.1493)
S Africa 1989	vs NZ 1988	0.0181	(0.0204)
S Aust 1988	vs S Aust 1989	0.0793	(0.0082)
S Aust 1988	vs W Tas 1988	0.1897	(0.1151)
S Aust 1988	vs NZ 1988	0.1096	(0.0210)
S Aust 1989	vs W Tas 1988	0.2433	(0.1021)
S Aust 1989	vs NZ 1988	0.1185	(0.0417)
W Tas 1988	vs NZ 1988	0.2004	(0.1434)

The mtDNA diversity of the 107 orange roughy individuals analysed with four-base enzymes was $0.6652 \pm 0.1335\%$ (mean $\pm$ standard error). The mean intrapopulational diversity results are summarised in Table 6.

Pairwise comparisons of all seven samples revealed nine net mtDNA-diversity estimates which were not significantly different from zero (Table 7). The remaining 12 pairwise comparisons yielded mean net mtDNA-diversity estimates greater than their corresponding standard errors, indicating possible genetic subdivision between these localities. The New South Wales sample consistently displayed a significantly different mtDNA diversity estimate compared to every other locality (Table 7). Out of the six pairwise mtDNA-diversity estimates involving the New Zealand sample, only two comparisons (South Africa vs New Zealand and western Tasmania 1988 vs New Zealand) were non-significant. There was also evidence of temporal differences in mtDNA diversity between Tasmanian samples of orange roughy and fish sampled in South Australian waters (eastern Tasmania 1988 vs South Australia 1989, diversity $= 0.0235 \pm 0.0083\%$ and South Australia 1989 vs western Tasmania 1988, diversity $= 0.2433 \pm 0.1021\%$). There was also an indication of a strong temporal difference between fish sampled in South Australian waters in 1988 and 1989 (diversity $= 0.0793 \pm 0.0082\%$, Table 7).

Discussion

The survey of the mitochondrial genome of *Hoplostethus atlanticus* with enzymes recognizing sequences of six nucleotides failed to detect any geographic structuring of mtDNA haplotypes. However, the greater resolving power of four-base enzyme analysis produced evidence, from both gene diversity and nucleotide-diversity analyses, of at least two stocks of orange roughy in southeastern Australian waters. There is strong evidence that orange roughy found off the coast of New South Wales are largely reproductively isolated from those found in South Australian and Tasmanian waters. Further sampling would be required to determine the position of the genetic break between the localities.

The results of the four-base enzyme analysis of orange roughy mtDNA support biological evidence suggesting that orange roughy in New South Wales waters are reproductively isolated from fish occurring further south. Bell et al. (1992) have shown that female orange roughy from New South Wales waters mature at 28 cm (SL) whereas females from South Australia and western and eastern Tasmania mature at 32 cm (SL). Further evidence of stock separation has been provided by the discovery of marked differences in the spawning times of orange roughy. Fish caught in New South Wales waters commence spawning 6 wk earlier than orange roughy in eastern Tasmanian spawning aggregations. Furthermore, the relative fecundity of the New South Wales fish is higher (Bell et al. 1992).

The genetic structure of orange roughy off the coast of South Australia and Tasmania is not well defined. There was no evidence of genetic separation amongst orange roughy sampled from South Australian and Tasmanian waters in 1988 (Table 7). However, orange roughy collected from the same localities off South Australia in 1989 were distinct from those collected in 1988, as well as from

orange roughy collected off western and eastern Tasmania in 1988 (Table 7). The availability of detailed biological, reproductive and catch-rate data for orange roughy caught southeast of Kangaroo Island makes it possible to put forward a scenario for the presence of genetically distinct samples in the same locality during consecutive years. The orange roughy specimens collected southeast of Kangaroo Island in 1988 were taken from large aggregations which were believed to be feeding aggregations (Newton 1988). These feeding aggregations did not reform in 1989. There is some evidence from the lower catch rates (Newton and Burnell 1989, Bulman et al. 1993) and reproductive condition of the fish sampled in 1989 (Bell et al. 1992) to suggest that the 1989 catches were principally composed of smaller pockets of non-breeding fish that were missed during the 1988 survey as a result of being numerically overwhelmed by the transient feeding aggregation.

There is also some evidence of genetic separation among southeastern Australian samples of orange roughy and fish sampled from New Zealand waters. Only one pairwise comparison between the New Zealand sample and the five Australian samples (Table 7) produced a non-significant result. However, there was no evidence of genetic separation between the west and east coasts of Tasmania. There was also no evidence to suggest that orange roughy sampled from southwestern African waters were genetically distinct from fish collected in southeastern Australian and New Zealand waters, except for one pairwise comparison (New South Wales 1988 vs South Africa 1989, diversity $=0.0699 \pm 0.0313\%$, Table 7). The reason for this lack of genetic differentiation between Australian and South African samples of orange roughy remains unclear. However, it is possible that successive generations of larvae spawned off South Africa may be transported eastward aided by westwind drift and the large anti-cyclonic gyres present in the Indian Ocean (Heath 1985). Alternatively, it is possible that the apparent lack of geographical structuring of orange roughy mtDNA haplotypes in the southern hemisphere and the associated low level of mtDNA diversity may be the result of a recent founder event. Elliott and Ward (1993) recorded a high level of genetic diversity (12% average heterozygosity per locus) in the nuclear genome of orange roughy which is not reflected in the mitochondrial genome of this species. Because the mitochondrial genome is haploid and clonally transmitted through the female lineage, the effective population size is one-quarter that of nuclear genes (Wilson et al. 1985). Therefore, mtDNA diversity is more seriously affected by founder events or population bottlenecks than the nuclear genome. Additional sampling and the use of a greater number of four-base enzymes may be needed to determine if any genetic structuring exists among orange roughy south of New South Wales.

A possibly limited dispersal capability of adult orange roughy may account for the apparent reproductive isolation of the New South Wales sample. There is some evidence from observations made in New Zealand that orange roughy may travel up to 200 km to reach a spawning area (Bell et al. 1992). It remains unclear as to how far orange roughy in Australian waters migrate in order to aggregate at a spawning or feeding ground. Circumstantial evidence of orange roughy migrations off the east coast of Tasmania has arisen from observed changes in the proportion of non-reproductive fish, due to the movement of mature individuals just prior to spawning (Bell et al. 1992). However, the pattern of migration is not well understood.

Although information on the larval biology of orange roughy remains incomplete, it appears that the greatest potential for dispersal in this species possibly occurs during the zygote and, to a lesser extent, the larval phase. Pankhurst et al. (1987) reported that fertilized orange roughy eggs are pelagic and ascend to surface waters following spawning. During this time the zygotes can, potentially, be carried considerable distances by ocean currents. However, T. Koslow (CSIRO Division of Fisheries, personal communication) suggests that orange roughy eggs may occur mainly between 700 and 900 m and are rarely found at shallower depths.

Reproductive isolation may be maintained between Tasmanian and New South Wales samples of orange roughy as the East Australian current (EAC) diverts eastward toward New Zealand at $\sim 33°S$. The remainder of the EAC continues to flow southward in a series of eddies toward Tasmania (Heath 1985). It is possible that the orange roughy larvae, if they are restricted to deeper waters as Koslow (personal communication) suggests, may not be trapped in these eddies and therefore are not transported southward toward Tasmania. Furthermore, the north-easterly flow of the EAC may contribute to the separation of New South Wales and New Zealand stocks of orange roughy due to larvae being carried well north of New Zealand (Hamilton 1990). However, because of the presence of the same common six-base haplotype and three rare haplotypes in both South Australian and New South Wales waters, the possibility of some mixing of eggs and/or larvae between both regions cannot be ruled out. Brasher et al. (1992) suggested that the characteristic flow of the EAC may be a contributing factor to the possible genetic separation of Australian and New Zealand populations of the palinurid lobster *Jasus verreauxi*. In an allozyme study of the calanoid copepod *Metridia pacifica*, Bucklin et al. (1989) identified nine polymorphic loci that were useful in distinguishing coastal samples of *M. pacifica* from offshore samples collected along the Californian coast. The genetic structure of *M. pacifica* was maintained by a cool coastal filament/offshore jet extending from the northern coast of California.

Allozyme studies of orange roughy in southeastern Australian waters have provided conflicting results. Black and Dixon (1989) used starch gel electrophoresis to study nine polymorphic enzyme loci in southeastern Australian samples of orange roughy. Their results suggest three subpopulations: South Australia, eastern Australia (including Tasmania) and New Zealand. Elliott and Ward (1993) undertook a more comprehensive allozyme study of orange roughy in southern Australian waters. They found that the allele frequencies at two polymorphic loci (mannosephosphate isomerase and malate de-

hydrogenase) are different from those reported by Black and Dixon. Furthermore, they found no evidence of genetic subdivision amongst the southeastern Australian samples of orange roughy and no evidence of deviation from Hardy-Weinberg equilibrium. The reason for the different results obtained by the two studies remains unclear. However, as illustrated by White and Shaklee (1991), inconsistent results may be obtained by different laboratories carrying out electrophoretic analyses on exactly the same samples. White and Shaklee recommend the testing of replicate samples in order to minimise such inconsistencies in electrophoretic data. Nevertheless, there are some possible biological factors that may explain apparent genetic differences amongst samples collected from approximately the same localities. For example, different migration patterns of orange roughy from year to year may be a contributing factor to the observed differences.

A study of the parasite fauna of orange roughy by Lester et al. (1988) identified five Australian stocks and three New Zealand stocks of this species. The Australian stocks were the Great Australian Bight, South Australia/west Victoria/west and south Tasmania, Cascade Plateau/Tasman Rise, northeast Tasmania and New South Wales. The multivariate analysis of the eight parasite categories examined by Lester et al. clearly demonstrated that parasites from small and medium-sized orange roughy sampled from New South Wales were distinct from other localities. Apart from the Great Australian Bight and the Cascade Plateau, the other localities tended to be more similar in terms of their parasite faunas.

Although parasite tags are a useful means of monitoring the movements of individual fish, it is seldom that parasite data can serve to delineate stock boundaries (Lester 1990). The parasite analysis of orange roughy cannot rule out the possibility that the five Australian "stocks" are derived from the same spawning population (Lester et al. 1988, Lester 1990). Nevertheless, Lester (1990) argues that parasite analysis has been more successful than genetic analyses in identifying these 'semi-isolated' stocks of orange roughy. It is possible that there are "semi-isolated" stocks of orange roughy south of New South Wales. However, the genetic analyses used in the present study failed to identify these as distinct stocks, due possibly to the homogenising effect of migration of larvae and/or adult fish between localities.

A study of the otolith microchemistry of orange roughy has suggested the presence of three "stocks" in southern Australian waters (Edmonds et al. 1991). This conclusion was drawn from the microchemical composition of whole otoliths taken from adult fish. Therefore, it cannot be ruled out that adult fish sampled from the three "stocks" may be derived from a single spawning population, as these results give no indication of the movements of juvenile fish. An analysis of the microchemistry of the otolith primordium would provide a clearer indication of whether separate breeding stocks of orange roughy occur in southeastern Australian waters.

The majority of mtDNA studies carried out on marine fish have used six-base rather than four-base enzymes for mtDNA analysis (Ovenden 1990). The polyacrylamide gel electrophoresis (PAGE) technique which is used in four-base enzyme analysis is technically more difficult to prepare than the agarose gel system used in six-base enzyme analysis. Furthermore, the amount of data generated by four-base enzyme analysis makes restriction-site analysis and enzyme mapping very difficult. A typical polyacrylamide gel is illustrated in Fig. 4. The mtDNA on this gel was prepared using the phenol/chloroform extraction method (Chapman and Powers 1984, as modified by Ovenden et al. 1989) which, if performed carefully, can yield mtDNA similar in quality to caesium chloride-purified mtDNA. A number of studies have demonstrated that six-base enzymes can nevertheless resolve the population structure of marine fish (Avise et al. 1987b, *Opsanus tau* and *O. beta*; Dahle 1991, *Gadus morhua*; Wirgin et al. 1989, *Morone saxatilis*). In some instances, however, six-base enzyme analysis may not be sensitive enough to detect much of the genetic variability present within a sample. In cases where the nucleotide diversity of a sample is known to be $<0.2\%$, four-base enzymes will be more informative than six-base enzymes (Ovenden 1990).

The level of mtDNA nucleotide sequence diversity in orange roughy identified with six-base enzyme analysis (0.12 to 0.19%) was low compared with diversity estimates obtained in mtDNA studies of other species of vertebrates (Ovenden 1990).

The level of intrapopulational nucleotide-sequence diversity in southeastern Australian orange roughy is lower than that found in the New Zealand and South African samples (Table 2). Furthermore, there is evidence of a decline in the level of nucleotide diversity which is particularly evident in eastern Tasmanian samples collected in consecutive years. In an allozyme study of nine polymorphic loci, Smith et al. (1991) reported a decrease in heterozygosity in samples of orange roughy collected off the west and east coast of New Zealand between 1982 and 1988. They attribute this decrease in heterozygosity directly to fishing activity. Smith et al. suggest that the older, arguably more heterozygous fish, tend to stay longer in the spawning aggregations that are targeted by the fishing industry, resulting in a higher mortality than less heterozygous fish. This explanation may be appropriate for the changes observed in the level of heterozygosity in the nuclear genome. However, there is no evidence from mtDNA analysis that older orange roughy exhibit a higher nucleotide-sequence diversity than younger fish. It is unlikely that this decline in mtDNA diversity is a consequence of a rapid decrease in the long-term effective population-size of orange roughy, mediated by fishing pressure, because of the longevity of the fish. Furthermore, because of the large standard errors associated with the diversity estimates in Table 2, it is possible that these estimates may have been calculated by chance. Alternatively, different stocks of orange roughy with correspondingly different levels of genetic diversity may have been sampled from the same collection sites during consecutive years. The higher levels of nucleotide sequence diversity found in the New Zealand and South African samples (Table 2) may reflect a larger effective popula-

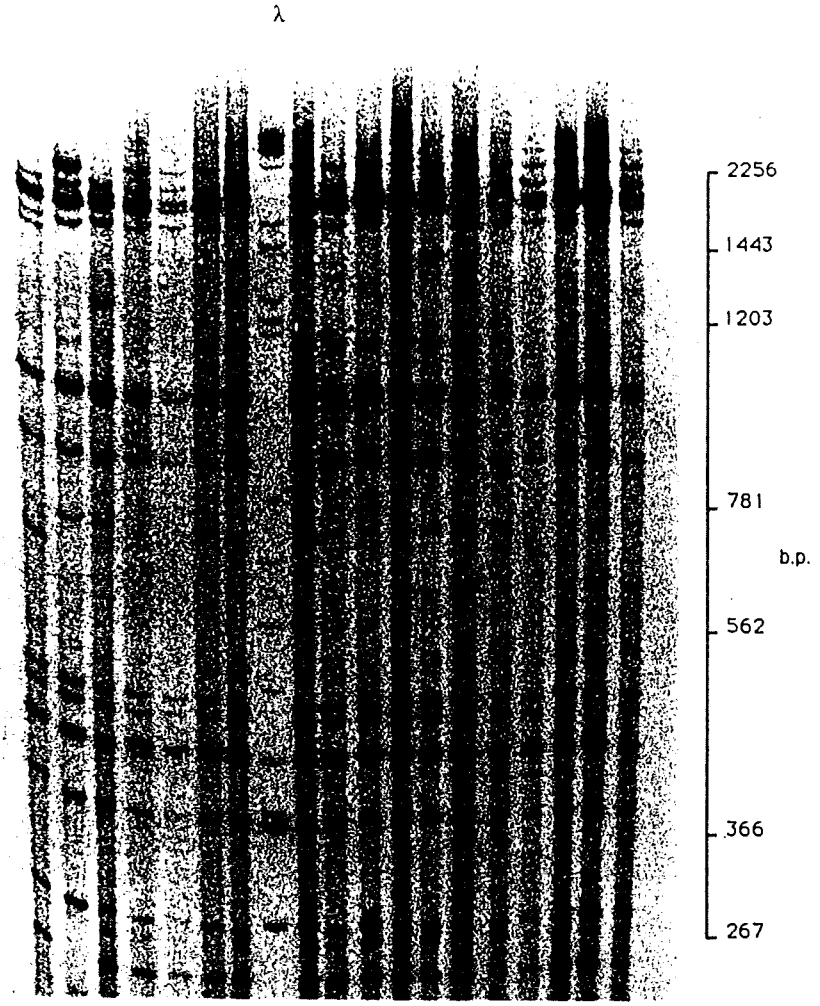

Fig. 4. *Hoplostethus atlanticus*. An autoradiograph of 18 orange mtDNA samples from Kangaroo Island (South Australia) cleaved with the enzyme *Taq*I, and bacteriophage lambda size-standard cleaved with the enzyme *Bgl*I (Lane 8 form left). Size scale with fragment sizes in base pairs (b.p.) is shown on right. The faint bands on the autoradiograph were more visible when viewed at a light source, and were also scored

tion size. The trends evident in the six-base intrapopulational diversity results (Table 2) are not supported by the four-base intrapopulational diversity results (Table 6). However, because the six-base results are based on site data and the four-base on fragment data, care should be taken when making comparisons.

The difference in the magnitude of the mtDNA-diversity estimates obtained with six-base and four-base enzyme analysis may be due to nucleotide sampling errors. Lynch and Crease (1990) demonstrated that nucleotide sampling (or the choice of restriction enzymes) and population sampling are responsible for most (in many cases > 90%) of the sampling variance for estimates of nucleotide diversity. The variance due to haplotype sampling is usually quite small.

The presence of discrete stocks of orange roughy in southeastern Australian waters has major implications for the management of this resource. Factors such as the long-lived nature of orange roughy (Fenton et al. 1991), intermittent spawning (Bell et al. 1992) and the possibly low vagility of adult fish, make this species susceptible to over-fishing. Further nucleotide and population sampling will be required in order to make confident conclusions about the population structure of orange roughy in southeastern Australian waters. The results of the four-base enzyme analysis of orange roughy mtDNA suggest that eastern Tasmanian and New South Wales samples are largely reproductively isolated. Further sampling of orange roughy from localities intermediate between Tasmania and New South Wales would be required in order to determine which model of population structure best describes this species.

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APPENDIX F

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Preliminary Investigation of Mitochondrial DNA Variation in Jack Mackerel (*Trachurus declivis*, Carangidae) from South-eastern Australian Waters

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Abstract

A preliminary investigation of the genetic structure of jack mackerel (*Trachurus declivis*, Carangidae) in south-eastern Australian waters was conducted by using restriction enzyme analysis of mitochondrial DNA (mtDNA). Seventy-five jack mackerel samples were collected from two localities: off Eden in New South Wales and off south-eastern Tasmania. Fish were sampled twice at each locality. Mitochondrial DNA was extracted from developing ovary tissue and heart tissue. The 75 jack mackerel samples were screened with 11 six-base and four four-base enzymes. The level of genetic diversity in jack mackerel was low. The six-base enzyme analysis found limited evidence of reproductive isolation between New South Wales and Tasmanian samples of jack mackerel. However, an analysis of a subset of 42 jack mackerel with four four-base enzymes found evidence of temporal differences in the mtDNA diversities of two Tasmanian samples of jack mackerel.

Extra keywords: population genetics.

Introduction

The fishery for jack mackerel (*Trachurus declivis*, Carangidae) is the largest single-species fishery in Australia, with a purse-seine fishing venture aimed exclusively at this species (Williams *et al.* 1987). The fish are used as a protein source for fish meal as well as for crayfish bait and, to a lesser extent, in tinned pet food (Williams *et al.* 1987). There are no reliable estimates of the abundance of jack mackerel in south-eastern Australian waters.

Jack mackerel are distributed along the entire southern coast of Australia and occur in all Tasmanian coastal waters (Stevens and Hausfeld 1982; Williams and Pullen 1986). Fish in south-eastern Australian waters mature at approximately 27 cm fork length and grow to a maximum length of 46 cm (Webb and Grant 1979; Williams and Pullen 1986). Stevens and Hausfeld (1982) calculated the theoretical maximum age of jack mackerel to be approximately 15 years.

Spawning occurs from late spring to mid summer in south-eastern Australian waters and tends to peak during January (Jordan 1992). Jack mackerel are thought to spawn along the entire eastern coast of Tasmania, with high egg densities being reported along the edge of the continental shelf (Jordan 1992). In south-eastern Australian waters, they feed primarily on crustaceans, with a particular preference for euphausids (Webb 1976). Young *et al.* (1993) found that the euphausid *Nyctiphanes australis* tended to be the dominant prey item in the stomachs of *T. declivis*, especially during the start of the fishing season in autumn. In a study of the feeding ecology of larval jack mackerel, Young and Davis (1992) found that crustacean microzooplankton and occasionally bivalve veligers were the main prey items.

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An allozyme study of jack mackerel in Australian waters by Richardson (1982) identified eight polymorphic loci that were useful in determining the stock structure of this species. That study identified a distinct subpopulation of jack mackerel in Western Australian waters. However, in the case of the south-eastern Australian fish, clear stock delineation was not evident although seven of the eight polymorphic loci revealed an excess of homozygotes.

The present paper describes the use of restriction enzyme analysis of mitochondrial DNA (mtDNA) as a means of determining the genetic structure of jack mackerel in south-eastern Australian waters. The rapid rate of evolution of the mitochondrial genome (Brown *et al.* 1979) and the clonal inheritance of this molecule through the maternal lineage (Avise 1987) make it a sensitive means of investigating genetic differentiation at the intraspecific level (Ovenden 1990). Restriction enzyme analysis of mtDNA has been useful in resolving the genetic structure of a number of species of marine fish, including orange roughy (*Hoplostethus atlanticus*; Smolenski *et al.* 1993), striped bass (*Morone saxatilis*; Wirgin *et al.* 1988) and cod *Gadus morhua*; Dahle 1991).

The jack mackerel fishery is a relatively recent development, and important data on the biology of this species are only now coming to light (Jordan 1992; Williams and Pullen 1993). It is anticipated that the potentially high resolving power of mtDNA analysis may provide a refined picture of the genetic structure of jack mackerel in south-eastern Australian waters, thereby contributing to an informed management policy.

Materials and Methods

Trachurus declivis was collected from two sites along the south-eastern Australian coast: off Eden in New South Wales (NSW) and off Eaglehawk Neck in Tasmania. The NSW samples were collected from two localities by the Department of Primary Industry and Energy, Bureau of Rural Resources, in November 1988; initially, fish were caught within a 10-km radius of 36°20′S,150°14′E and referred to as NSW Sample 1 (NSW1). The following day, fish were collected within a 20-km radius of 36°56′S, 150°18′E (NSW2), using mid-water trawls (between 100 and 120 m depth). The samples from Eaglehawk Neck (43°1·5′S,147°55′E) were collected with a beach seine in January 1989 and April 1990.

For mtDNA analysis, 29 fish were chosen arbitrarily from the two NSW samples (NSW1, $n=14$; NSW2, $n=15$) and 46 fish were chosen from the Tasmanian 1989 ($n=30$) and 1990 ($n=16$) samples. The length of the NSW fish was $33\cdot7\pm1\cdot6$ cm (mean ± s.d., $n=29$) and the weight was $449\cdot0\pm57\cdot7$ g (mean ± s.d., $n=29$). The length of the fish from the Tasmanian 1989 sample was $33\cdot2\pm1\cdot7$ cm (mean ± s.d., $n=30$). The length and weight data for the Tasmanian 1990 sample are unavailable.

Mitochondrial DNA was extracted from developing ovary and heart tissue by using a phenol/chloroform extraction method described by Chapman and Powers (1984).

The jack mackerel genomes were screened with 11 six-base enzymes. A subset of 42 individuals was screened with four four-base enzymes. The conditions for the six-base and four-base enzyme assays have been described previously (Smolenski *et al.* 1993).

The restriction sites recognized by analysis with six-base restriction enzymes were mapped upon the jack mackerel mitochondrial genome by using the single- and double-digest technique described previously (Smolenski *et al.* 1993). The fragment sizes were converted to number of base pairs mapped clockwise from the *Bgl*II site (Fig. 1). The mapping of the jack mackerel genome was aided by the use of a computer package written for this purpose (Ovenden *et al.* 1992).

The observed and expected six-base and four-base haplotype frequencies were compared by using a χ^2 test. A Monte Carlo method (Roff and Bentzen 1989) was used to test the significance of the χ^2 values, which were compared with the values obtained from 1000 randomizations of each data set.

The G statistic (G_{st}) was used to estimate the amount of interpopulational genetic subdivision of jack mackerel samples and is equal to the amount of variation in the whole data set that is due to the division of the population into a series of interbreeding populations (Takahata and Palumbi 1985). Restriction-site presence or absence data in binary form were used to test the six-base enzyme data, whereas restriction fragment data were used for the four-base enzyme analysis. The significance of using site versus fragment data is discussed in Smolenski *et al.* (1993). A bootstrap method (Palumbi and Wilson 1990) was used to evaluate the significance of the G_{st} analysis.

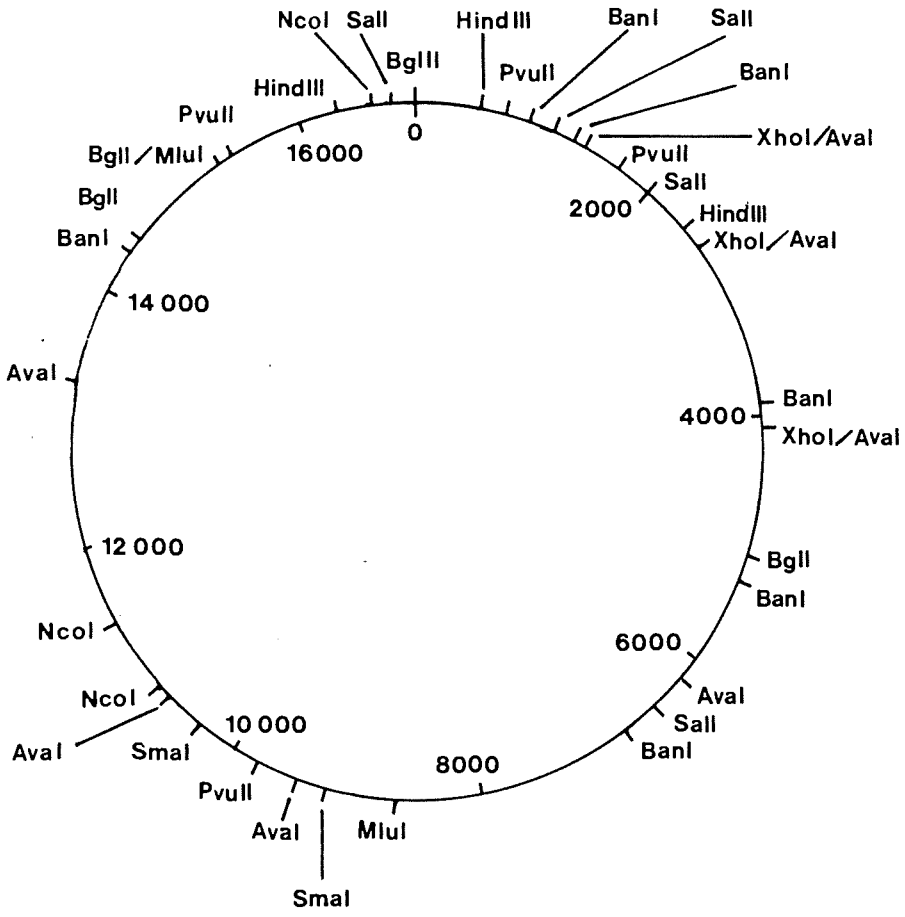

Fig. 1. Map of the jack mackerel mitochondrial genome (common haplotype AAAAAAAAAAA). Restriction sites are mapped clockwise from the *Bgl*II site. A size scale for the restriction sites (in base pairs) is shown around the inside of the map.

The number of base substitutions per base pair (∂) between pairs of individual fish and between pairs of composite six-base enzyme haplotypes was calculated by using the maximum-likelihood method of Nei and Tajima (1983). The calculation of ∂ from the presence or absence of four-base restriction fragment data is described by Nei and Li (1979). The standard deviation of ∂ was calculated according to Nei and Tajima (1983) (Smolenski *et al.* 1993).

The method for calculating the mean net ∂ between populations and the group variance of ∂ is described by Nei and Jin (1989). Student's *t*-test was used to determine whether the net diversity estimates were significantly different from zero (Smolenski *et al.* 1993). However, because the pairwise comparison of net diversity estimates between localities is not statistically independent, a sequential Bonforroni test (Rice 1989) was carried out on significant net nucleotide diversity estimates. The level of significance, α (where $\alpha = 0{\cdot}05$), was adjusted by using this method to obtain a corrected significance level, α'.

Results

Six-base Enzyme Survey

An estimate of the size of the mitochondrial genome in jack mackerel was obtained by averaging the sum of all the fragments for each haplotype, resulting in a value of 16 432 ± 460 base pairs (mean ± s.d.).

The restriction enzyme survey of 75 jack mackerel genomes with 11 six-base enzymes identified 48 restriction sites. Of the 11 restriction endonucleases used to survey the mitochondrial genome of jack mackerel, four enzymes (*Nco*I, *Pvu*II, *Sal*I and *Sma*I) identified no restriction site variation. The remaining restriction endonucleases identified between one and five different morphs each. Of the 75 genomes surveyed, *Ban*I produced five variant morphs and *Ava*I identified three different morphs. Five enzymes (*Bgl*I, *Bgl*II, *Hin*dIII, *Mlu*I and *Xho*I) identified one rare morph each: *Bgl*II, *Hin*dIII, *Mlu*I and *Xho*I each produced one rare morph that had gained one restriction site in comparison with the common morph, whereas the rare morph identified by the enzyme *Bgl*I differed from the common morph by one restriction site loss. The three rare morphs (B, C and D) identified by *Ava*I had each gained one restriction site in comparison with the common morph. The enzyme *Ban*I identified three rare morphs (B, E and F) that differed from the common morph by one restriction site gain, whereas the rare morphs C and D had both lost a restriction site in comparison with the common morph (Fig. 2).

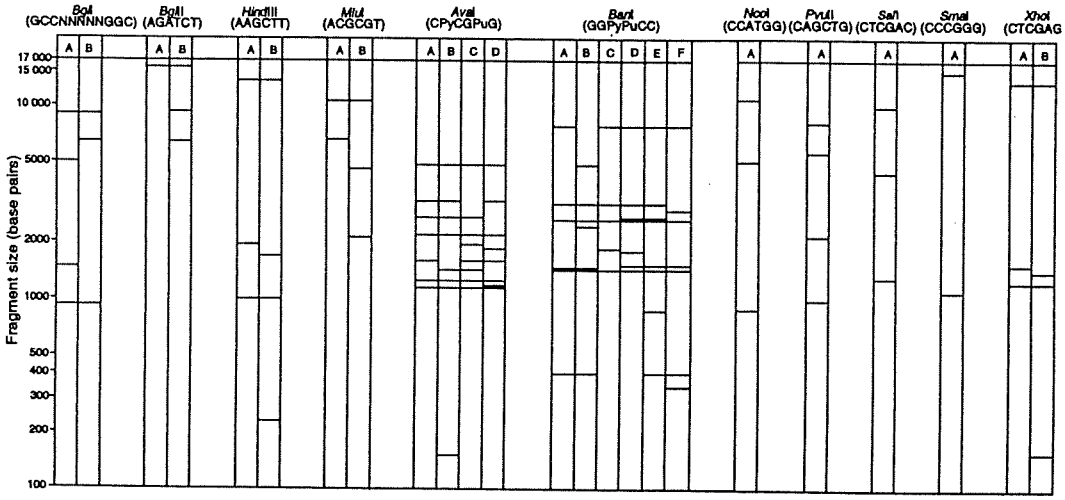

Fig. 2. Fragment patterns for jack mackerel mitochondrial haplotypes identified by 11 six-base restriction enzymes. Recognition sequences of the restriction enzymes are in parentheses.

The six-base restriction enzyme survey identified 12 mitochondrial haplotypes. The most common haplotype (AAAAAAAAAAA) was recorded in 22 of 29 individuals collected in NSW waters and in 36 of 46 fish collected in Tasmanian waters. The fish from NSW waters were characterized by seven unique haplotypes, each represented by one individual. Among the fish from Tasmanian waters, 10 individuals possessed five rare haplotypes. The rare haplotype AEAAAAAAAAA was found in six individuals exclusively from Tasmanian waters (Table 1). Apart from the common haplotype (AAAAAAAAAAA), only one rare haplotype (AAABAAAAAAA) was recorded from both NSW and Tasmanian waters (Table 1).

The amount of mtDNA restriction site variation that was due to population subdivision (G_{st}) calculated from the presence or absence of restriction sites was 37%. This estimate lies within the range of G_{st} estimates calculated from 1000 randomizations (bootstrapped G_{st}) of the original data set (29% to 39%) and may not, therefore, indicate population subdivision.

The χ^2 test was used to test the null hypothesis that there is no geographical partitioning of jack mackerel six-base enzyme haplotypes. The resulting χ^2 value, tested for significance by the Monte Carlo method (Roff and Bentzen 1989), produced a non-significant result ($P \leqslant 0{\cdot}053 \pm 0{\cdot}014$).

Table 1. Geographical distribution and number of jack mackerel haplotypes identified by six-base enzyme analysis

NSW1, New South Wales Sample 1; NSW2, New South Wales Sample 2; T.89, Tasmanian 1989 sample; T.90, Tasmanian 1990 sample. The order of the 11 enzymes in the composite haplotypes is *Ava*I, *Ban*I, *Bgl*I, *Bgl*II, *Hin*dIII, *Mlu*I, *Nco*I, *Pvu*II, *Sal*I, *Sma*I and *Xho*I

Haplotype	NSW1	NSW2	T.89	T.90	Total
AAAAAAAAAAA	8	14	22	14	58
AAABAAAAAAA	1	0	1	0	2
ACAAAAAAAAA	0	1	0	0	1
AEAAAAAAAAA	0	0	5	1	6
AEAABAAAAAA	1	0	0	0	1
AFAAAAAAAAA	1	0	0	0	1
BABAAAAAAAB	1	0	0	0	1
ABAAAAAAAAA	1	0	0	0	1
ADAAAAAAAAA	1	0	0	0	1
CAAAAAAAAAA	0	0	1	0	1
DAAAAAAAAAA	0	0	1	0	1
AAAAABAAAAA	0	0	0	1	1
Total	14	15	30	16	75

The mtDNA diversity, or mean number of base substitutions per nucleotide, for all jack mackerel individuals analysed with six-base enzymes in this study was $0{\cdot}1363 \pm 0{\cdot}0723\%$ ($n = 75$). Intrapopulational diversity estimates are summarized in Table 2.

Table 2. Mean intrapopulational mtDNA diversity estimates (%) and standard errors (%) for four jack mackerel populations analysed with six-base and four-base enzymes

Analysis and population	Diversity	s.e.	*n*
Six-base analysis			
New South Wales (Sample 1) 1988	0·2962	0·1236	14
New South Wales (Sample 2) 1988	0·0314	0·0334	15
Eastern Tasmania 1989	0·1654	0·1031	30
Eastern Tasmania 1990	0·0286	0·0305	16
Four-base analysis			
New South Wales (Sample 1) 1988	0·3652	0·1298	8
New South Wales (Sample 2) 1988	0·3077	0·1137	10
Eastern Tasmania 1989	0·6263	0·1279	16
Eastern Tasmania 1990	0·5235	0·1432	8

Four-base Enzyme Survey

Four restriction enzymes recognizing sequences of four bases were used to survey a subset of 42 individuals selected from the 75 genomes surveyed with six-base enzymes (NSW Sample 1, $n = 8$; NSW Sample 2, $n = 10$; Tasmanian 1989 Sample, $n = 16$; Tasmanian 1990 sample, $n = 8$). The four four-base enzymes produced 160 restriction fragments for the four enzymes. The enzymes *Dde*I and *Taq*I identified 13 and 12 different restriction morphs, respectively. The enzyme *Mbo*I identified 19 unique restriction morphs, and *Msp*I was the most variable enzyme, identifying 28 unique restriction morphs.

The four-base analysis of 42 jack mackerel genomes identified 36 unique composite mitochondrial haplotypes (Table 3). There was no common haplotype, and only three four-base haplotypes (AAAA, ABAA and ABCA) occurred more than once (Table 3).

Table 3. Geographical distribution and number of jack mackerel four-base haplotypes

NSW1, New South Wales Sample 1; NSW2, New South Wales Sample 2; T.89, Tasmanian 1989 sample; T.90, Tasmanian 1990 sample. The order of the four enzymes in the composite haplotypes is *Dde*I, *Mbo*I, *Msp*I and *Taq*I

Haplotype	NSW1	NSW2	T.89	T.90	Total
AAAA	1	0	1	0	2
AAB'B	1	0	0	0	1
AAQA	0	0	1	0	1
AASB	0	0	1	0	1
AAUC	1	0	0	0	1
ABAA	1	2	2	0	5
ABCA	0	2	0	0	2
ABDA	0	1	0	0	1
ABLA	0	0	1	0	1
ACXA	0	1	0	0	1
ACZA	0	1	0	0	1
ADHE	0	0	1	0	1
ADIB	0	0	1	0	1
AERA	0	0	1	0	1
AHGA	0	0	1	0	1
APBA	0	0	1	0	1
BAA'B	1	0	0	0	1
BAAA	0	1	0	0	1
BAAB	1	0	0	0	1
BACB	1	0	0	0	1
CABJ	0	0	0	1	1
CBAI	0	0	0	1	1
CFAA	0	0	0	1	1
CGBG	0	0	0	1	1
CMEA	0	0	0	1	1
DABA	0	1	0	0	1
DKKA	0	0	1	0	1
EIMH	0	0	0	1	1
FJJD	0	0	1	0	1
GCYK	0	0	0	1	1
HBFL	0	0	0	1	1
IEAC	0	0	1	0	1
JNOF	0	0	1	0	1
KOPA	0	0	1	0	1
LQEA	1	0	0	0	1
MRVA	0	1	0	0	1
Total	8	10	16	8	42

The bootstrapped G_{st} estimate for the four jack mackerel samples was 24%. The G_{st} values calculated from 1000 randomizations of the data set ranged from 16% to 22%. This result suggests population subdivision because the true G_{st} value (24%) was greater than the randomly generated G_{st} values.

The χ^2 tests comparing all localities were carried out for enzyme-specific morphs that occurred more than once. Enzyme-specific morphs that occurred only once were omitted in order to avoid inflated significance values. The null hypothesis being tested was that there was no geographical separation of the jack mackerel four-base morphs. Each χ^2 result was tested for significance by the Monte Carlo method (Roff and Bentzen 1989). Three enzymes

(*Dde*I, *Mbo*I and *Taq*I) yielded a significant result ($P \leqslant 0{\cdot}001$, $P \leqslant 0{\cdot}035 \pm 0{\cdot}011$ and $P \leqslant 0{\cdot}043 \pm 0{\cdot}0029$, respectively). The enzyme *Msp*I produced the only non-sigificant result ($P \leqslant 0{\cdot}056 \pm 0{\cdot}014$; Table 4).

Table 4. Geographical distribution and number of jack mackerel *Dde*I, *Mbo*I, *Msp*I and *Taq*I morphs that occurred more than once

Enzyme	Locality	Morph				
		A	B	C	D	E
*Dde*I	New South Wales Sample 1	4	3	0		
	New South Wales Sample 2	7	2	0		
	Eastern Tasmania 1989	14	0	0		
	Eastern Tasmania 1990	0	0	5		
*Mbo*I	New South Wales Sample 1	6	0	0	0	0
	New South Wales Sample 2	3	5	2	0	0
	Eastern Tasmania 1989	4	3	0	2	2
	Eastern Tasmania 1990	0	2	0	0	0
*Msp*I	New South Wales Sample 1	3	0	0		
	New South Wales Sample 2	4	0	2		
	Eastern Tasmania 1989	4	0	0		
	Eastern Tasmania 1990	2	2	0		
*Taq*I	New South Wales Sample 1	3	4			
	New South Wales Sample 2	10	0			
	Eastern Tasmania 1989	11	3			
	Eastern Tasmania 1990	2	0			

Table 5. Mean interpopulational mtDNA diversity estimates (%) and standard errors (%) for four jack mackerel populations analysed with four-base enzymes
Underlined numbers represent possibly significant values

Population comparison	Diversity	s.e.
NSW Sample 1 1988 *v.* NSW Sample 2 1988	0·0296	0·0182
NSW Sample 1 1988 *v.* eastern Tasmania 1989	0·0193	0·0149
NSW Sample 1 1988 *v.* eastern Tasmania 1990	0·1147	0·0224
NSW Sample 2 1988 *v.* eastern Tasmania 1989	0·0058	0·0222
NSW Sample 2 1988 *v.* eastern Tasmania 1990	0·0861	0·0315
Eastern Tasmania 1989 *v.* eastern Tasmania 1990	0·0770	0·0087

The mtDNA diversity for all the jack mackerel individuals analysed with four-base enzymes was $0{\cdot}5153 \pm 0{\cdot}1035\%$ ($n = 42$). The intrapopulational mtDNA diversity estimates are summarized in Table 2. The net mtDNA diversity estimates between jack mackerel genomes from pairs of localities ranged from 0·1147% to 0·0058% (Table 5). Student's *t*-test was used to test whether the net diversity estimates were significantly different from zero. In three of these comparisons, the magnitude of the standard error was considerably smaller than the magnitude of the estimate (NSW Sample 1 versus eastern Tasmania 1990, $0{\cdot}1147 \pm 0{\cdot}0224\%$, $P < 0{\cdot}001$, $\alpha' = 0{\cdot}01$; NSW Sample 2 versus eastern Tasmania 1990, $0{\cdot}0861 \pm 0{\cdot}0315\%$, $P < 0{\cdot}01$, $\alpha' = 0{\cdot}0125$; eastern Tasmania 1989 versus eastern Tasmania 1990, $0{\cdot}0770 \pm 0{\cdot}0087\%$, $P < 0{\cdot}001$, $\alpha' = 0{\cdot}008$; Table 5), indicating possible genetic separation between the eastern Tasmanian 1990 sample and the remaining three samples. When both Tasmanian samples of jack mackerel were combined and compared with the combined NSW samples, the resulting net divergence estimate was not significant (diversity $= 0{\cdot}0142 \pm 0{\cdot}0165\%$).

Discussion

The six-base analysis of jack mackerel mtDNA found limited evidence of geographical structuring of mitochondrial haplotypes on the basis of the occurrence of a rare haplotype (AEAAAAAAAAA; Table 1) exclusively in Tasmanian waters. However, this preliminary evidence for stock separation among the jack mackerel samples is not supported by statistical and sequence diversity analysis. The use of enzymes recognizing sequences of four bases found no clear separation of mtDNA haplotypes between the Tasmanian and NSW samples. However, the four-base analysis did provide evidence of genetically distinct schools of jack mackerel occurring off eastern Tasmania in different years (Table 5). Nucleotide sampling errors (or the choice of restriction enzymes) and possibly small sample sizes may explain why the six-base enzyme analysis partitioned heterogeneity in the data in a different way from the four-base analysis. The choice of restriction enzymes may explain the difference in the magnitude of the mtDNA diversity estimates obtained with six-base and four-base enzyme analyses (Table 2) (Smolenski *et al.* 1993).

Care should be taken when making comparisons between six-base and four-base data because the former are based on site data and the latter on fragment data. Six-base enzymes have been useful in determining the genetic structure of a number of species of marine fish (Wirgin *et al.* 1988; Dahle 1991). However, in some cases, six-base enzyme analysis may lack the resolving power to detect much of the genetic variability present within a sample. It has been suggested that if the nucleotide diversity of a sample is less than 0·2%, four-base enzymes are more informative than six-base enzymes (Ovenden 1990). An analysis of mtDNA variation in the deep-sea teleost *Hoplostethus atlanticus*, using four-base enzymes, revealed evidence of reproductive isolation, whereas previous six-base enzyme analysis failed to identify any geographical structuring of mtDNA haplotypes (Smolenski *et al.* 1993).

The presence of genetically distinct schools of jack mackerel occurring off eastern Tasmania lends support to the findings of Richardson's (1982) allozyme study. Richardson found two distinct subpopulations of jack mackerel in Western Australia and New Zealand; the population structure of jack mackerel in south-eastern Australian waters was less clearly defined. Five enzymes (carboxylesterase, isocitrate dehydrogenase, aconitate hydratase, L-iditol dehydrogenase and adenosine deaminase) all revealed an excess of homozygotes in fish collected from south-eastern waters. This excess of homozygotes was presumed to be the result of a Wahlund effect (i.e. the presence of two or more overlapping but genetically distinct groups in a population).

The underlying assumption from Richardson's results is that jack mackerel maintain a relatively strong school fidelity for most of their lives, with only very limited gene flow occurring between schools. However, in a study of the schooling behaviour of jack mackerel, Williams and Pullen (1993) suggest that jack mackerel targeted by fishers primarily form feeding schools especially in the presence of large concentrations of the euphausid *Nyctiphanes australis*. This suggests that, in the absence of zooplankton prey species, the jack mackerel may disperse rather than remain as discrete schools. In the 1989–90 fishing season, there was a marked decline in the abundance of *N. australis*, thought to be the result of a La Niña event (Pullen *et al.* 1989). Consequently, the jack mackerel did not school in coastal waters and instead were caught in deeper water adjacent to the fishing grounds.

Sharp (1978) suggested the possibility of sibship within schools of tuna (*Thunnus* spp.) on the basis of the results of an allozyme study. In the present study, the differences in the intrapopulational diversity estimates between the jack mackerel samples from NSW waters and those from Tasmanian waters suggest that there may be fidelity within schools (Table 2). Further genetic analysis would be required to test for sibship within schools of jack mackerel. Owing to the possible mixing of larvae by ocean currents following spawning, it seems unlikely, however, that jack mackerel would form sibling cohorts. The possibility that groups of the serranid reef fish *Anthias squamipinnis* were settling as sibling cohorts

was tested by Avise and Shapiro (1986), using allozyme electrophoresis. They found no evidence of sibship; instead, juvenile cohorts of *A. squamipinnis* were found to be composed of offspring from several matings.

A morphometric analysis of jack mackerel from Australian waters also found evidence of stock separation between fish from the Great Australian Bight and NSW waters (Lindholm and Maxwell 1988). However, no separation was detected between fish from NSW and Tasmanian waters.

Jack mackerel are known to spawn along the entire eastern coast of Tasmania, with spawning activity being concentrated at the edge of the continental shelf (Jordan 1992). The larvae then move toward inshore areas. Jordan (1992) suggests that interannual variation in the local hydrography off eastern Tasmania may affect the onshore movement of jack mackerel eggs and larvae, resulting in higher mortality rates. Fish, such as jack mackerel, that inhabit pelagic waters have the benefit of the greater productivity of surface waters but are also subject to greater predation pressure (Mead *et al.* 1964). In a study of the causes of mortality in North American jack mackerel (*Trachurus symmetricus*), Hewitt *et al.* (1985) found that the principle cause of mortality in yolk-sac larvae was predation. However, starvation was the major cause of mortality in feeding larvae once they had absorbed their yolk sacs. There are no data on the predators of jack mackerel eggs and larvae in Australian waters. It is possible that the loss of large numbers of larval jack mackerel to a combination of poor or no advection to inshore areas and predation may adversely affect subsequent recruitment, resulting in the possible extinction of some mtDNA lineages. Such losses would have the effect of reducing the effective population size of the spawning aggregations, thereby contributing to a lower overall mtDNA diversity. However, the physical and biological factors that determine the pattern of recruitment in jack mackerel remain unclear.

The ability of modern purse-seine vessels to remove almost entire schools during a single netting operation may also have a serious long-term effect on the mtDNA diversity of jack mackerel. In an allozyme study of orange roughy (*Hoplostethus atlanticus*), Smith *et al.* (1991) argued that fishing pressure was directly responsible for a loss of heterogeneity in that species.

The present study has presented some evidence to support Richardson's (1982) view that there may be a number of genetically distinct schools of jack mackerel occurring along the south-eastern coast of Australia. However, an expanded mtDNA study involving further nucleotide and population sampling will be required to determine the population structure of jack mackerel in south-eastern Australian waters.

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