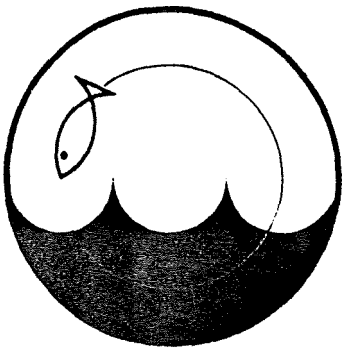
STOCK IDENTIFICATION AND DISCRIMINATION OF MULLOWAY IN AUSTRALIAN WATERS. (FIRTA 86/16)

FINAL REPORT

By M. Black and P.I. Dixon.



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SUMMARY OF MAIN FINDINGS

The population structure of mulloway throughout Australian waters was investigated with a view to providing a basis for management decisions for this recreationally and commercially important fishery. This study is limited by two factors:-

1/ rapid deterioration of tissue samples, affecting both allozymes and mtDNA,

and 2/ small sample sizes from key sites and no samples available from Victoria.

Allozymes

Four polymorphic loci were used as genetic markers. Six localities around Australia were sampled. These collections consisted, in the main, of juvenile fish. We found evidence of a separate subpopulation in W.A. with discontinuity in allele frequency at two loci, Adh and Sdh. At two loci, Adh and Aat, alleles rare in the Coorong, S.A. were found to be more prevalent in N.S.W., so the possibility of further population substructuring is indicated. Fish sampled from the West Coast of S.A. were more similar to N.S.W. fish than those sampled from the Coorong, S.A. Replicate year class samples were taken from the Coorong, S.A. and the Hawkesbury River, N.S.W. At two loci, Ada and Sdh, for the Coorong fish, and at all loci for the N.S.W. fish, the replicate samples were significantly different. These differences were as large as the differences found between localities. In all cases rarer alleles became more prevalent in the later samples.

Isoelectric Focusing

The soluble general muscle proteins were examined using IEF. We found differences in the minor bands within mulloway, both between and within different localities. However these differences are not useful for determining population structure. This technique is a very useful tool for distinguishing species with differences in the major protein bands. Of the other Sciaenid species analysed in this way, we found one mis-identified specimen. These other species were not present in any of our collections.

Restriction enzyme digests of mtDNA

This method was found to be unsuitable for mulloway due to the poor quality of the mtDNA obtained. Two restriction enzymes were found to have a a recognition sequence in mulloway, namely Ava1 and Hind111.

Direct sequencing of mtDNA

A section of approximately two-thirds of the cytochrome b gene was successfully sequenced for six fish from N.S.W., and this sequence compared to two fish from S.A., one from the Coorong and the other from the West Coast.

No sequence variation was found either within or between these localities. These results contradict those obtained using allozymes, and indicate panmixia across this geographic range. We were unsuccessful in sequencing the W.A. allozyme samples, and further specimens from W.A. should be sought. The D-Loop failed to amplify, so no sequence was obtained for this region of mtDNA.

RECOMMENDATIONS

The allozyme data suggest that mulloway found in W.A. waters are genetically distinct from the rest of the fishery. However further work needs to be completed before firm conclusions may be drawn that may be of use to those making management decisions about the mulloway fishery. We recommend:-

1/ Further sampling be undertaken at suitable localities between S.A. and W.A. to determine the extent of these two putative subpopulations.

2/ Any future allozyme studies undertaken should also include Pgm from liver (see text for explanation).

The mtDNA sequence work was of a preliminary nature. It is essential to clarify the population substructuring suggested by the allozyme data. This would best done by completing further sequencing work with:-

1/ more fish (already obtained) from S.A., to be sequenced in the cytochrome b gene,

2/ further attempts to obtain sequence from the D-loop region by investigating alternative primers,

and 3/ further specimens from W.A.

ACKNOWLEDGEMENTS

During the course of this study there have been many people willing to offer their various skills to facilitate the smooth running of the programme. We are most grateful to them all. Deserving of special mention are our successful collectors.

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INTRODUCTION

Mulloway (*Argyrosomus hololepidotus*) in Australian waters is an important commercial and recreational fish. The largest commercial catches are taken in New South Wales and South Australia. Catches have, however, been declining in South Australia in recent years. The reasons for this are complex, but it appears that the decline is related to lowered outflows from the Murray River coupled with high fishing pressure from both recreational and commercial fishermen.

In South Australia the key question is the relationship between the mulloway on the east and west coasts:-

1. Are they separate stocks?

or 2. Do they belong to one large interbreeding population?

Because mulloway is a large, strongly swimming fish with a wide distribution, and because of its increasing importance as a table fish, it is appropriate that the stock question be addressed over the whole distributional range of the species in Australia.

The aim of this project was to investigate the population structure of *Argyrosomus hololepidotus*, with the view to determining whether mulloway in Australian waters belong to one large interbreeding population throughout their range, or whether two or more separate stocks exists. This information is of considerable relevance to future management decisions for this species.

The methods used in this study for stock identification were:-

1. electrophoresis of enzymes,
2. isoelectric focusing of soluble muscle proteins,
3. analysis of restriction digest fragments of mitochondrial DNA,

and 4. direct sequencing of selected regions of the mitochondrial genome.

The initial objective in our programme was to investigate the population structure of mulloway using allozymes as genetic markers. Selected locations within the geographic range of the species were sampled by netting, trawling and angling, with the co-operation of Fisheries Officers in the different states, and commercial and amateur fishermen.

However, in cases where electrophoretic surveys of fish species fail to provide evidence of significant genetic variation, or where large sample sizes are difficult to obtain, restriction fragment pattern analysis of mitochondrial DNA (mtDNA) may be of value. This provides more detailed information of population structure, examining variation at the nucleotide (rather than gene product) level.

We proposed to use restriction digest fragment patterns of mtDNA to supplement the allozyme electrophoretic analysis of mulloway population structure. Two problems arising during the allozyme study that pointed to mtDNA being a better technique for this species were:-

1. a low level of suitable allozyme polymorphism,

and 2. small sample sizes from most of the collection sites.

For mtDNA, sample sizes of 20 fish from each site (approximately 160 fish across the entire range of the species) would be sufficient. Statistical analysis of allozyme data requires sample sizes of approximately 100 animals from each site (for large populations) to be reliable. Restriction fragment pattern analysis of mtDNA is a much more sensitive technique for the determination of variation. Several different protocols were used to isolate sufficient supercoiled mtDNA for restriction digestion to be of value. However, none proved suitable for mulloway.

Mitochondrial DNA is a rapidly evolving extranuclear genetic system. It is clonally and maternally inherited in all animals. For these reasons its inheritance pattern may be considered as that of a single, haploid gene. Nucleotide sequences of stretches of mtDNA can be used to construct gene trees (a phylogeny of haplotypes or alleles for any specified stretch of DNA). The maternal transmission of mtDNA effectively makes this gene tree a matriarchal phylogeny (Avise, 1989), allowing us to trace the history of mtDNA through and between populations and species. Matriarchal trees can be good estimators of population and species trees due to mtDNA's lower effective population size (Crozier, 1990).

The relationship between a phylogeny of haplotypes and their geographic distribution has been termed phylogeography (Avise *et al.*, 1987). Its use in determining population structures and demographies, both current and historical, is rapidly advancing.

In this research two regions of mtDNA were examined. Sections of the cytochrome b gene and the D loop region were selected to be amplified using two pairs of "universal primers" (Kocher *et al.*, 1989) and the polymerase chain reaction (PCR). The amplified templates were then directly sequenced. These two regions were chosen because of their different evolutionary rates. The cytochrome b gene has an intermediate rate of evolution (compared to other mtDNA genes) while the D loop is the most rapidly evolving region of mtDNA.

METHODS

Sample collection

Considerable time and effort was directed towards the collection of specimens. However, we had limited success in obtaining the large numbers of specimens required for electrophoresis. We were unsuccessful in obtaining a large sample from northern NSW. A collecting trip to Shark Bay (WA), conducted by the WA Fisheries Research Laboratories, was disrupted by cyclonic weather. The only sample of 80 fish collected in Victoria was not sent to the laboratory due to a freezer failure there. Further attempts to obtain more samples from Western Australia and Victoria were unsuccessful.

Table 1 lists the numbers, sex ratio and the size range of fish collected from each locality. Figure 1 is a map indicating these sampling sites. Mulloway collected for electrophoretic screening were sent frozen whole to the laboratory. Tissue specimens of liver, heart, muscle and kidney (for pilot screen only) were dissected from partially thawed fish and placed into 1.2ml NUNC tubes. For mtDNA analysis, tissue samples of liver and (where appropriate) ripening female gonad were dissected from fish within 1 hour of capture and placed immediately into a liquid nitrogen dewar supplied for transportation to the laboratory. All samples were stored cryogenically (-180^{0}C to -196^{0}C) until required for electrophoresis or mtDNA preparation.

Allozyme electrophoresis

Full details of the electrophoretic procedures used in this study follow the methods described in the whiting stock identification report (Dixon *et al.*, 1986). Starch gels were made from 12%(w/v) Electrostarch (Lot Number 87). Prior to the commencement of population comparisons a pilot study was carried out following the strategy described by Richardson *et al.*, (1986). Forty enzyme systems were surveyed for genetic variation in mulloway using horizontal starch gel electrophoresis in three different buffer systems. Ten fish each from NSW and WA, and four tissues (liver, heart, kidney and muscle) were used to determine which enzymes were polymorphic. Tissue samples were divided and homogenised in either an equal volume of either deionized/distilled water, or reducing buffer (pH8) to search for optimum resolution on the gels. Those loci which were polymorphic ($P_{0.99}$) formed the basis of population comparisons. Appendix 1 lists the enzymes surveyed for genetic variation, and Appendix 2 details the electrophoresis running conditions and presumed number of loci for each tissue. Thirty-seven enzyme systems showed activity, representing 56 presumptive loci.

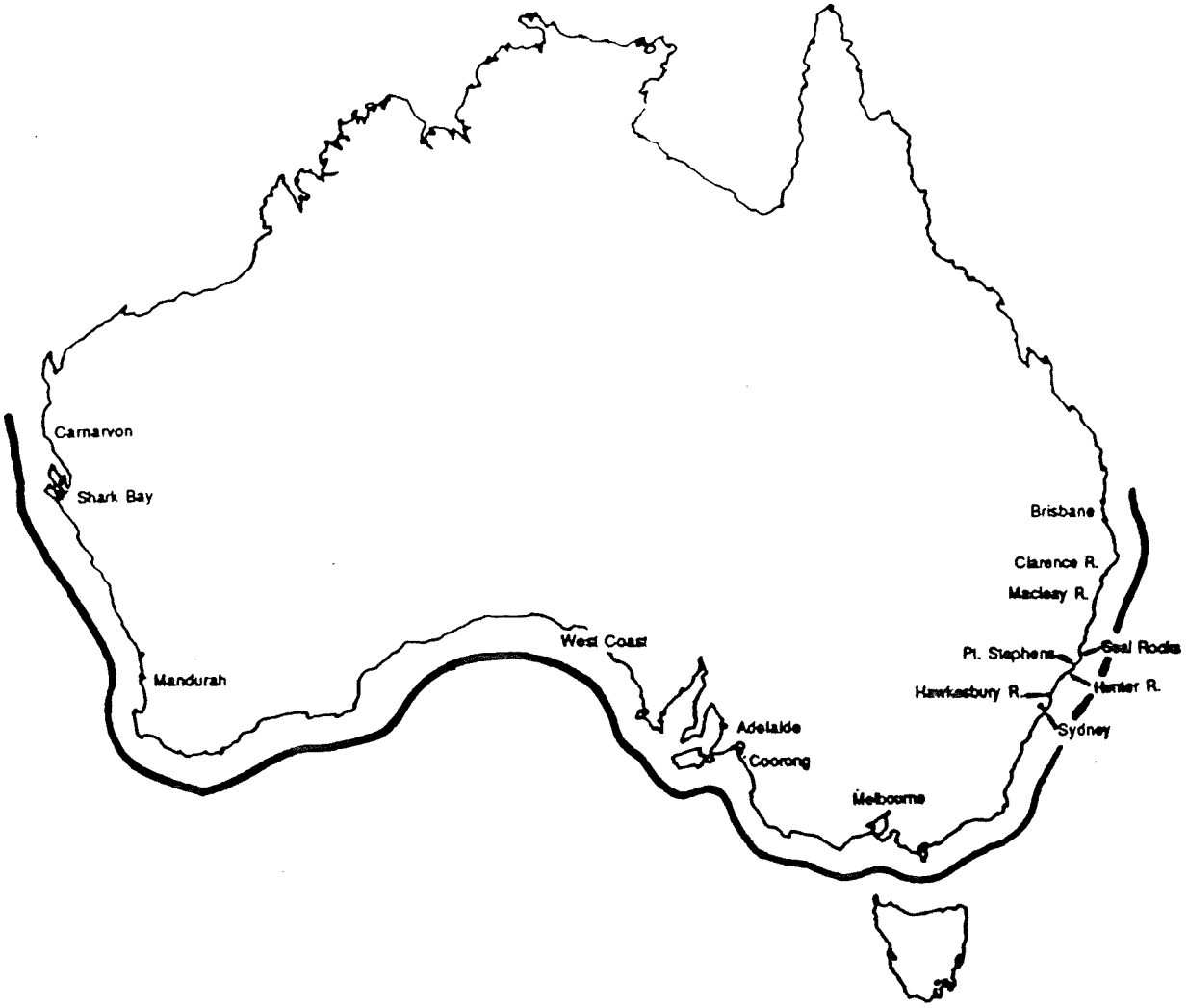


Figure 1 Map to show the collection sites and distribution of mulloway (*Argyrosomus hololepidotus*) around Australia.

Table 1. Collection data for *Argyrosomus hololepidotus*.

Collection Site	Collection Date	No. of Animals	Sex Ratio	Size Range SL (cm)	Weight Range (kg)
NSW					
Macleay R.	9/86	12[a]	-	52-112	1.8-19.8
Clarence R.	9/86	3[a]	-	96-115	7.4-14
Seal Rocks	2/88	1[b]	1F	-	18
Port Stephens	2/88	9[b]	6F:3M	96-120	-
Hunter R.	11-12/88	125	125J	8-30	-
Terrigal	2/88	1[b]	1F	-	25
Hawkesbury R.	11/86	147	147J	13-38	-
	8/87	44	44J	8-36	-
Sydney area	9/86	13[a]	-	55-107	2.2-14.6
	3/87	1[b]	1M	80	-
Bondi	11/'88	1[b]	1F	-	-
Cronulla	1/88	3[b]	3F	-	9.2-19.3
SA					
Coorong	4/86	21	1M:20J	18-48	-
	5/86	85	85J	18-38	-
	2/87	136	4F:1M:131J	14-33	-
	8/87	6[b]	3F:1M:2J	-	7-10
	11/87	18[b]	-	27-42	-
	11/87	73	73J	16-34	-
West Coast	12/86	36	1F:2M:13J	-	1.3-27.8
	8/87	2[b]	2F	64-65	-
	12/87	8[b]	7F:1M	97-137	-
	12/88	3[b]	3F	-	-
WA					
Mandurah	1/87	30	4F:26J	21-42	-
	2/87	2	-	38-36[c]	-
Shark Bay (in)	5/87	1	-	47[3]	-
(out)	5/87	13	-	62-73[c]	-
Carnarvon	5/87	30	30J	24-34[c]	-

Key: [a] = muscle samples only obtained from Sydney Fish Markets, [b] = samples for mtDNA, [c] = total length, F = female, M = male, J = juvenile, SL = standard length, - = no data.

Fifteen enzyme loci were selected for use as genetic markers to identify stocks of mulloway. These were:- Adh, Sdh, Mpi and Pep(LGG) from liver tissue, Ada, Pgm-1, Pgm-2, Gpi-1, Gpi-2 and Mdh-2 from muscle tissue, and Aat-2, Est-2, Idh-1, Idh-2 and Pep(PL) from heart tissue. Three buffer systems (Poulik, TM pH 7.8 and CAM pH 6.1) were chosen to optimise resolution on the starch gels. Progress was hampered by rapid deterioration of tissue samples.

Appendix 3 describes the enzyme banding patterns observed for each of these loci. Eleven loci were not included in the statistical analysis programs either due to:-

1. variable staining, caused probably by post-transcriptional modifications (Est-2, Idh-1, Idh-2, Mdh-2, and Mpi),

or 2. an extremely low level of polymorphism, (Gpi-1, Gpi-2, PepLGG, PepPL, Pgm-1, and Pgm-2).

Table 2 lists the enzyme loci regarded as suitable for statistical analysis.

The choice of muscle tissue to screen PGM, with hindsight, was unfortunate as the polymorphisms detected in Pgm-2 from liver tissue in the pilot study were subsequently found not to be reflected in Pgm-2 from muscle. Therefore the possible population difference between WA and NSW indicated initially in the pilot screen (see Appendix 2) was not realised in the data obtained for this locus.

Table 2 Informative loci used for the comparison of *Argyrosomus hololepidotus* populations.

ENZYME	ABBREVIATION	EC NUMBER	TISSUE	BUFFER
Aspartate aminotransferase	Aat-2	2.6.1.1	heart	CAM pH 6.1
Adenosine deaminase	Ada	3.5.4.4	muscle	TM pH 7.8
Alcohol dehydrogenase	Adh	1.1.1.1	liver	Poulik
Sorbitol dehydrogenase	Sdh	1.15.1.1	liver	Poulik

Allozyme data analysis

Patterns of enzyme variation that were consistent with the known subunit structure of the enzyme (Shaklee and Keenan, 1986) were used for discrimination of sub-populations. Names of enzymes and Enzyme Commission numbers follow the recommendations of the Commission on Biochemical Nomenclature (Anon, 1984).

For multilocus enzyme systems, the form with the least anodal migration was designated "1", the next "2", and so on (in accordance with the recommendations of Allendorf and Utter, 1979). For each locus, alleles were indicated alphabetically, with the most anodally migrating allele designated "a", the next "b", and so on. For loci with cathodal migration, the most cathodally migrating allele was designated "a". The putative genotype data were tabulated as genotype and allele frequency distributions in a form suitable for input into the statistical programs described below (see Appendix 4).

We used Felsenstein's (1981, 1982) continuous character, maximum likelihood method for constructing phylogenetic trees from these data. The program CONTML (Version 2.7) is part of Felsenstein's PHYLIP package. A dendrogram was constructed from the output from CONTML using the program TreeDraw. Dendrogram construction provides valuable information on the inter-relationships of populations, but does not provide a test of whether pairs of populations are genetically distinct. The G-test (Sokal and Rohlf, 1981, pp 745-746) provides a simple yet powerful test for distinguishing populations, and uses all the gene frequency data available. The program POPSEP, written by us, performs G-tests on all possible pairs of populations. The Weighted Chi-squared (Sokal and Rohlf, 1981) program, written by us, tests for population variance at each locus weighted by degrees of freedom.

The genetic distance between pairs of populations was also used to construct phylogenetic trees. This was done, not because it is the most appropriate method, but because of the widespread application of these measures in electrophoretic studies of systematics (Hillis, 1984). The program NEIBOTH, written by us, computes Nei's genetic distance, D^* (as modified by Hillis, 1984) and identity, from the allele frequency distributions. We used Felsenstein's (1981, 1982) Fitch-Margoliash least-squares distance method for constructing phylogenetic trees from these data. The program FITCH (Version 2.7) is part of Felsenstein's PHYLIP package. A dendrogram was constructed from the output from FITCH using the program TreeDraw.

The genotype distributions of loci polymorphic at the $P_{0.90}$ level were examined for internal consistency with the Hardy-Weinburg distribution. Any deviations from Hardy-Weinburg equilibrium were then analysed using Smith's H statistic (Smith, 1970). Pie charts were produced from allele frequencies using the Macintosh program Cricket Graph.

Isoelectric focusing (IEF)

LKB Ampholine polyacrylamide gels (pH range 3.5 to 9.5) were used to separate soluble muscle proteins for a comparison of samples of mulloway (*Argyrosomus hololepidotus*) from three States:

-Shark Bay and Carnarvon, W.A.,

-the west coast and the Coorong in S.A., and

-the Hawkesbury and Macleay Rivers, N.S.W.

A few specimens of other species within the Sciaenidae were included in these comparisons, namely:- *Johnius vogleri*, *Johnius amblycephalus*, and *Austronibea oedogenys* (collected from the Gulf of Carpentaria). We did not find any of these species amongst our mulloway collections. Full details of the procedures followed are detailed in the whiting stock identification report (Dixon *et al.*, 1986).

Isoelectric focusing data analysis

We used Felsenstein's (1981,1982) mixed method parsimony to construct Wagner trees from the isoelectric focusing data. The program MIX (Version 2.7) is part of Felsenstein's Phylip package. The gels were scored as a series of two state characters ("1" and "0") to indicate the presence or absence of a band respectively. Further details about this program are detailed in the whiting stock identification report (Dixon *et al.*, 1986). A dendrogram was constructed from the output from MIX, using the Hypercard stack Treedraw Deck.

Mitochondrial DNA (mtDNA)

Collections of the smaller sample sizes for mtDNA analysis had been more successful. With the help of spearfishermen and local anglers we obtained specimens from various locations in NSW (from Sydney, north to Port Stephens and Seal Rocks). However, a sample of 4 fish collected from Iluka was lost due to the unexpectedly rapid evaporation of refrigerant from the flask (liquid nitrogen). In N.S.W. waters, mulloway are rarely caught south of Sydney, and (despite a number of attempts) we were unable to obtain any specimens from there. Collections were made for us from the mouth of the

Murray River, Coorong and the west coast of South Australia. Collections from Western Australia and Victoria were unsuccessful. (Refer to Table 1.) We did, however, utilise some of the replica tissue samples stored initially for electrophoresis for mtDNA sequencing.

Restriction fragment patterns of mtDNA

Two protocols were followed in an attempt to isolate mtDNA from mulloway:-

(i) the rapid method for fish used by Chapman and Powers, 1984.

and (ii) the caesium chloride density ultracentrifugation method used by Lansman *et al.*, 1981.

Full details of these methods, and the protocol followed for the restriction enzyme digestion and visualization of fragments, are detailed in Appendices 5 and 6.

A total of ten fish from seven localities were used:- West Coast S.A. (2 fish), Coorong, S.A. (2 fish), Seal Rocks, N.S.W. (1 fish), Port Stephens, N.S.W. (2 fish), Terrigal, N.S.W. (1 fish), Hawkesbury River, N.S.W. (1 fish), and Bondi, N.S.W. (1 fish). Tissue samples of liver and (where appropriate) maturing female gonad were stored in liquid nitrogen (-180^{0}C to -196^{0}C) until required for laboratory preparation. These tissues were requested as they had proven to be suitable in other fish species studied in this manner (eg orange roughy). For the one Bondi fish we used "fresh" tissue. In this case the fish was transported to the laboratory on ice. The organs were dissected from the animal and kept refrigerated until used, 30 hours after capture. We did not observe any appreciable difference in the amount of mtDNA obtained from these unfrozen samples. Heart was also used from this fish, and a slightly better yield of mtDNA was obtained using this tissue. Following this more encouraging result, we requested that heart tissue also be collected for mtDNA analysis.

This preliminary screening yielded no results because we were unsuccessful in finding a protocol suitable for mulloway.

Sequencing mtDNA

Following the failure of the above method we decided to directly sequence mtDNA. Sequence data was obtained by preparing crude extracts of total DNA from liver, muscle, heart or gonadal tissue. The crude extract was then subjected to the Polymerase Chain Reaction. PCR involves the amplification of stretches of DNA which are flanked by regions whose sequence is well known in the species, or in related species. Primers that are

complementary to these known sequences were prepared and used to amplify the region of interest. Sequencing was performed on single stranded PCR templates using the Sequenase® sequencing system.

A total of 19 individuals from seven localities were selected for sequencing:- Hawkesbury River, N.S.W. (5 individuals), Coorong, S.A. (4 individuals), West Coast S.A. (2 individuals), Cronulla, N.S.W. (2 individuals), Bondi, N.S.W. (1 individual), Terrigal, N.S.W. (2 individuals) and Carnarvon, W.A. (3 individuals). All mulloway tissues were transfered from liquid nitrogen at the end of the previous work (April, 1989) and stored in the ultracold freezer (-70^{0}C) until required for laboratory preparation.

(a) Crude isolation of total DNA

Approximately 0.2 grams of tissue (liver, muscle, heart, or gonad) was used. Tissue was frozen with liquid nitrogen and ground into a powder. The ground tissue was then added to 0.5ml homogenizing solution (NaCl, 100mM; Tris Cl, 10mM; EDTA, 1mM;) containing Proteinase K (10mg/ml, 25µl) and S.D.S. (10%, 50µl). Digestion of the samples was performed at 55^{0}C for no less than 1.5 hours. Samples were then phenol extracted twice (phenol: chloroform: isoamyl alcohol, 25:24:1) and traces of phenol removed by extracting with chloroform/isoamyl alcohol (24:1). DNA was precipitated with NaCl (5M, 20µl) and ethanol (95%, 1ml). DNA was precipitated overnight, at -20^{0}C. The pellets were then dried and resuspended in distilled water (between 100 and 300µl, depending on the size of the pellet).

(b) PCR amplificaton of the cytochrome b and D-Loop regions

The following pairs of primers were synthesized and purified:-

Cytochrome b1	5'-CCATCCAACATCTCAGCATGATGAAA-3'
Cytochrome b2	5'-CCCTCAGAATGATATTTGTCCTCA-3'

N.B.:- these primers span a region of approximately 307bp's in the cytochrome b gene and have been shown to amplify the region in many species of birds, mammals and other vertebrates (Kocher *et al.*, 1989).

D-Loop light strand	5'-TCAAGCTTACACCAGTCTTGTAAACC-3'
D-Loop heavy strand	5'-TAACTGCAGAAGGCTAGGACCAAACT-3'

N.B.:- these primers amplify about 1kb of the major non-coding region of mtDNA. It has been found to successfully amplify the region in most mammals and many fish (Kocher *et al.*, 1989).

The following protocols were used:

Double Stranded PCR:-

Total DNA (2µl) was added to a solution containing *Taq.* buffer (10X, 10µl), dNTP's (2.5mM, 8µl), primer 1 (10mM, 2µl), primer 2 (10mM, 2µl) and water (76µl). Samples were denatured at 93^0C for 5 minutes, then treated with *Taq* polymerase (2 units). The samples were then subjected to 35 cycles of PCR under the following conditions:- denature (92^0C/1 minute), anneal (53^0C/1minute), extend (72^0C/1 minute). Products were checked on a 1% agarose minigel, phenol extracted, and then precipitated with sodium acetate (1/10 volume) and 95% ethanol (2.5 volumes). Pellets were resuspended in 40µl deionized H_2O.

Single Stranded PCR:-

In single stranded PCR only one primer is used resulting in the production of single stranded DNA templates.

Double stranded amplified DNA (1µl) was added to *Taq* buffer (10X, 10µl), dNTP's (2.5mM, 8µl), primer (10mM, 1µl), and H_2O. Samples were denatured for 5 minutes/93^0C., then subjected to 35 cycles of PCR under conditions identical to those of double stranded PCR. Products were checked on a 1% agarose minigel and precipitated with ammonium acetate (7M, 1/3 volume) and 95% ethanol (4/3 volumes). Pellets were washed with 70% ethanol, dried and resuspended in 16µl H_2O.

Sequencing Single Stranded Amplication Products:-

Single stranded templates were sequenced directly on a 6% vertical polyacrylamide gel. All samples were treated as if they were double stranded DNA.

Primer Template Annealing:-

DNA template (7µl), 5X Sequenase® buffer (2µl) and primer (1µl) were heated to 94^0C for 5 minutes then quenched immediately in a dry ice/ethanol bath. Samples were then allowed to return slowly to room temperature in a pre-chilled (0^0C) dry bath block by which time primer template annealing should have been complete.

Labelling:-

Dithiothreitol (0.1M, 1µl), H_2O (2µl), alpha^{35}S dATP (0.5µl) and dilute Sequenase® enzyme (13U/µl,1:8 dilution in TE buffer, 2µl) were added to the primer template solution, mixed and incubated for 15 minutes at room temperature.

Termination:-

5µl of labelling mix was added to 2.5µl of the appropriate termination mixture (ddGTP, ddATP, ddTTP or ddCTP), mixed and incubated for 15 minutes at 37^0C. Reactions were stopped with 4µl Sequenase® stop solution. Reactions were left on ice until loaded. Immediately prior to loading samples were denatured at 95^0C for 5 minutes. Samples were loaded on a 6% vertical polyacrylamide gel and run for 1.5 hours at 2500V.

For further details on sequencing by vertical polyacrylamide gel electrophoresis, and hints on the crude isolation of mtDNA and PCR amplification, refer to Appendix 7.

RESULTS AND DISCUSSION

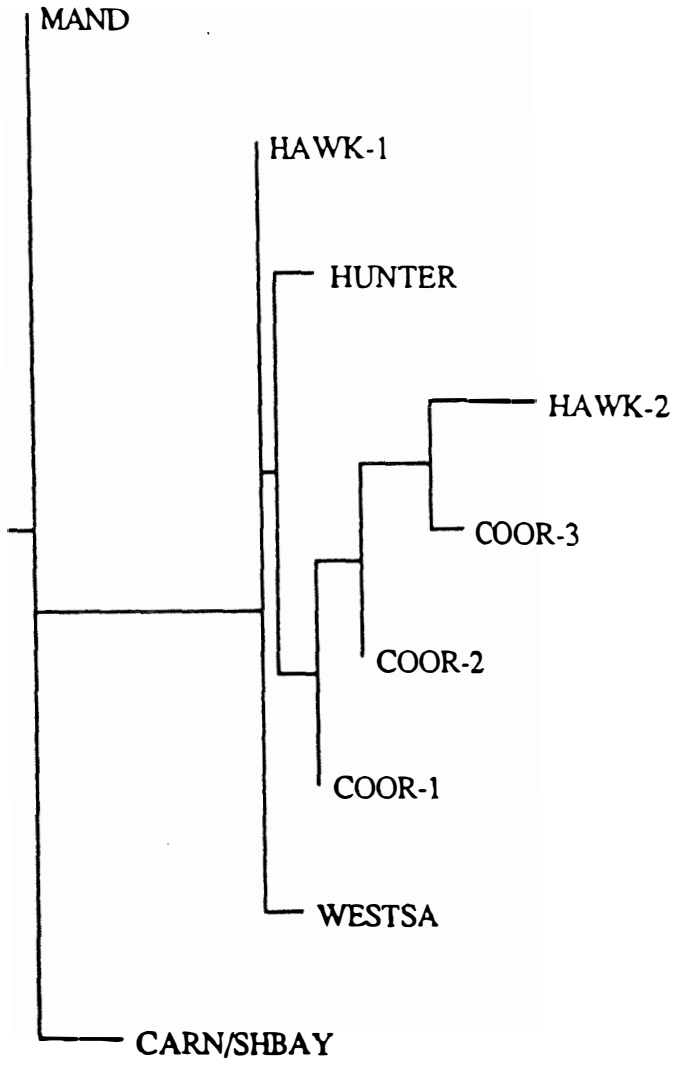
Allozyme electrophoresis

The large numbers of fish required for the electrophoretic study were collected over the two and a half year period from April, '86 through to December, '88. At five localities it was unfortunate that the sample size was small:- from Carnarvon, W.A. (30 fish), Shark Bay, W.A. (14 fish), Mandurah, W.A. (32 fish), the west coast of S.A. (36 fish), and Hawkesbury River, N.S.W. (44 fish). At some localities multiple samples were available. From the Hawkesbury River, N.S.W. samples were taken on two occasions (11/'86 and 8/'87), and from the Coorong, S.A. samples were taken on three ocassions (11/'86, 2/'87 and 11/'87).

The allele frequency data from six localities and nine sample collections, using four polymorphic loci, are given in Appendix 4, Table 4.1. The small collections taken at about the same time from Carnarvon and Shark Bay, W.A. were pooled. These data were repeatedly put into Felsenstein's CONTML program, changing the order of sites to find the best tree. The data output from CONTML for the tree of highest likelihood was then used to construct the dendrogram shown in Figure 2. Examination of the grouping of collection sites in the dendrogram show clustering according to geographic areas. The discontinuity (shown in the dendrogram by the greater branch length) of the Western Australian samples indicate the possible existence of two sub-populations of mulloway in Australian waters: one from South Australia to New South Wales, and another in Western Australia.

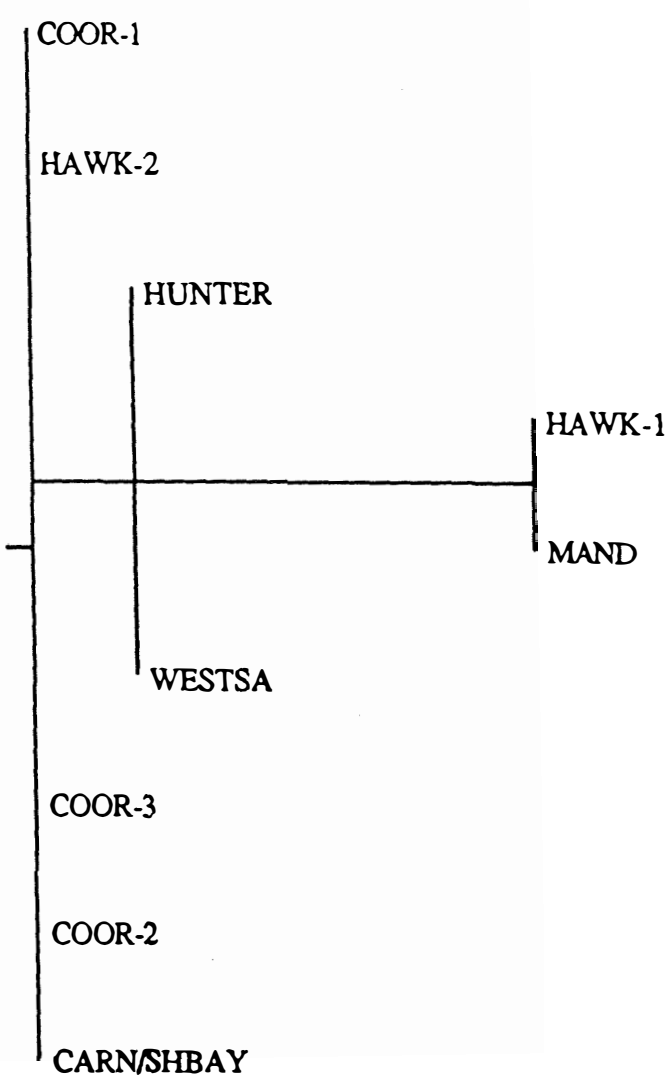
It is important to note that this information can only be regarded as tentative as it is based upon small sample sizes for three key sites investigated:- the two WA collections and the west coast of SA (see Appendix 4, Table 4.2). Figures 4, 5, 6, and 7 are maps showing pie charts of the allele frequencies at each locus. The geographic separation of the Western Australian fish is shown clearly at the Adh locus, with the alternate allele "c" common in both Western Australian collection sites and allele "b" common elsewhere (see Figure 4). Adh (Figure 8) and, to a lesser extent, Sdh (Figure 11) show this discontinuity in allele frequency as a stepped cline.

There is no evidence of clinal variation at the other loci examined. However, repeated collections over subsequent years show an increasing proportion of rarer alleles. In the Hawkesbury River, N.S.W. this phenomenon was observed across all four loci, and in the Coorong, S.A. this occurred at two loci, Ada and Sdh (see Figures 8 to 11).



Key: Carn/ShBay = Carnarvon,W.A. (5/'87) and Shark Bay, W.A. (5/'87) pooled; Mand = Mandurah, W.A. (1/'87); WestSA = West coast of S.A. (12/'86); Hawk-1 = Hawkesbury River,N.S.W. (11/'86); Hawk-2 = Hawkesbury River,N.S.W. (8/'87); Hunter = Hunter River, N.S.W. (11-12/'88); Coor-1 = Coorong, S.A. (5/'86); Coor-2 = Coorong, S.A. (2/'87); Coor-3 = Coorong, S.A. (11/'87).

Figure 2 Dendrogram to show the relationships between *Argyrosomus hololepidotus* populations (CONTML plot).



Key: Carn/ShBay = Carnarvon,W.A. (5/'87) and Shark Bay, W.A. (5/'87) pooled; Mand = Mandurah, W.A. (1/'87); WestSA = West coast of S.A. (12/'86); Hawk-1 = Hawkesbury River,N.S.W. (11/'86); Hawk-2 = Hawkesbury River,N.S.W. (8/'87); Hunter = Hunter River, N.S.W. (11-12/'88); Coor-1 = Coorong, S.A. (5/'86); Coor-2 = Coorong, S.A. (2/'87); Coor-3 = Coorong, S.A. (11/'87).

Figure 3 Dendrogram to show the relationships between *Argyrosomus hololepidotus* populations (FITCH plot).

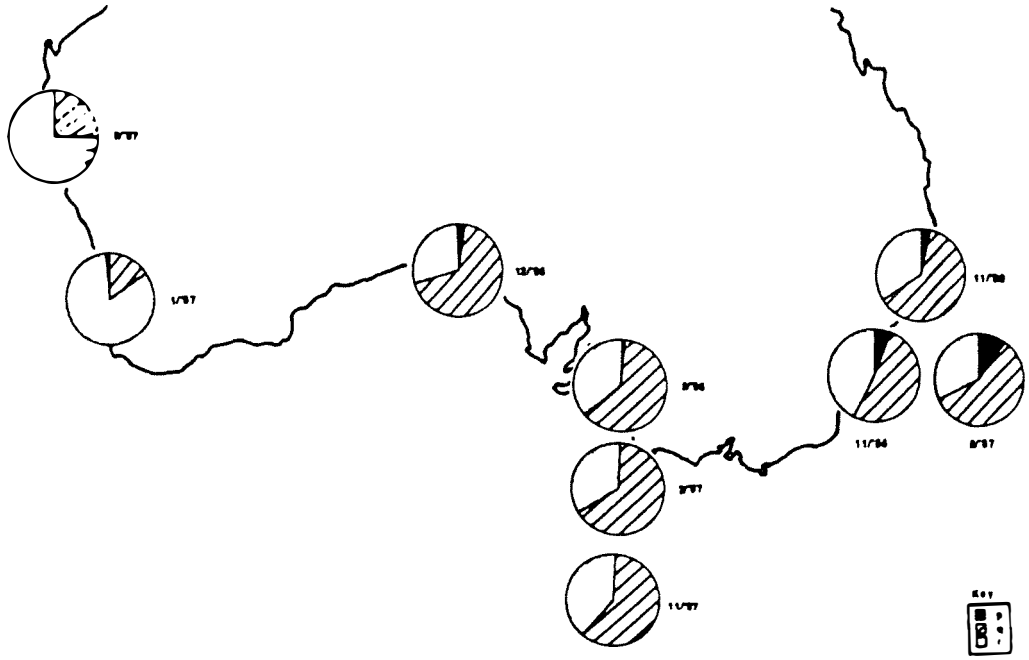


Figure 4 Map to show the frequencies as pie charts of Adh alleles from six localities around Australia, including 2 localities where more than one sample was taken.

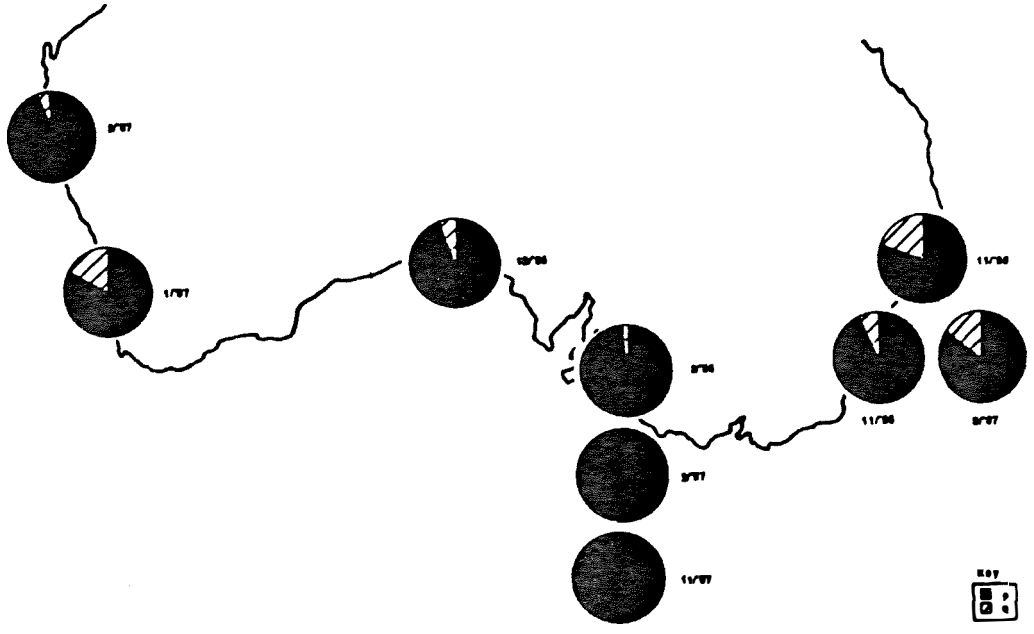


Figure 5 Map to show the frequencies as pie charts of Aat-2 alleles from six localities around Australia, including 2 localities where more than one sample was taken.

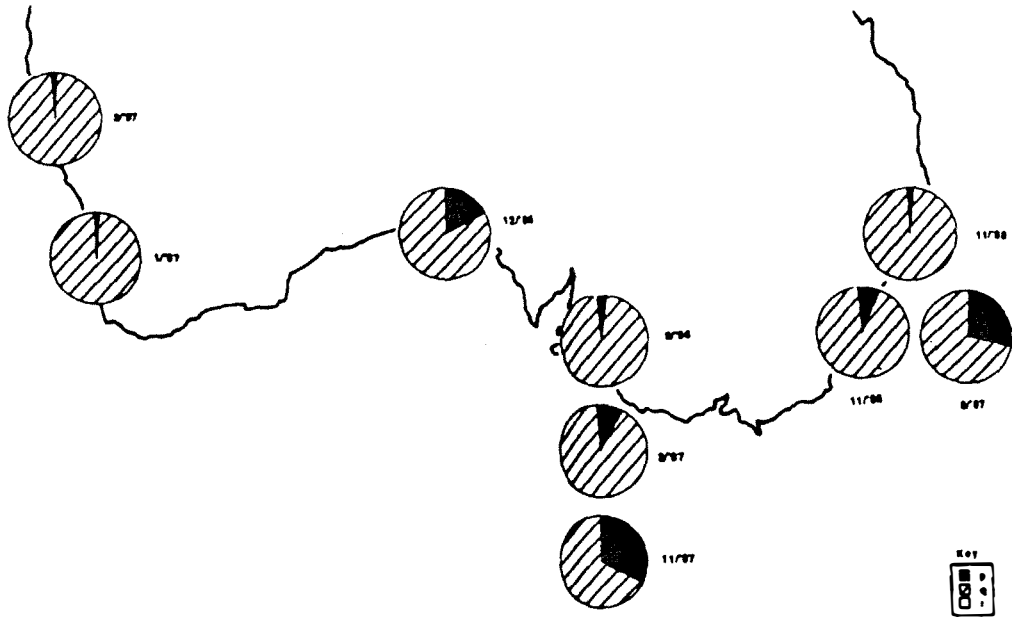


Figure 6 Map to show the frequencies as pie charts of Ada alleles from six localities around Australia, including 2 localities where more than one sample was taken.

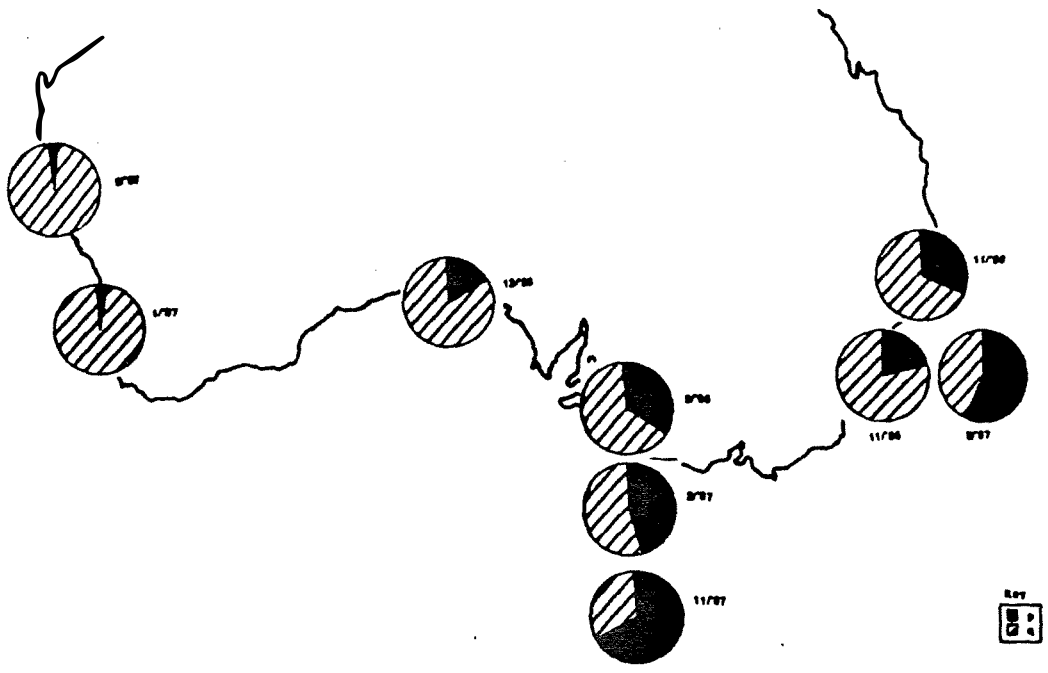


Figure 7 Map to show the frequencies as pie charts of Sdh alleles from six localities around Australia, including 2 localities where more than one sample was taken.

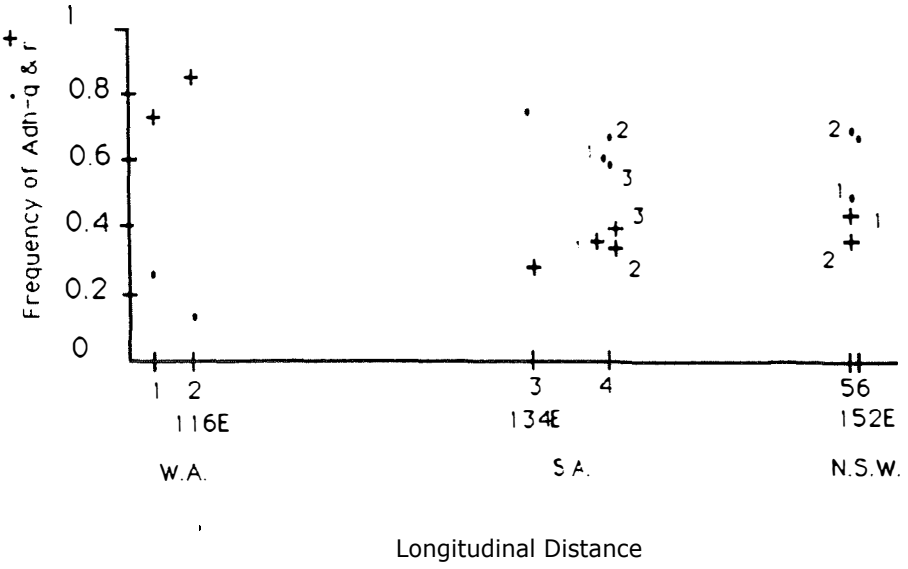


Figure 8 Scattergram of Adh q- and r-allele frequencies by longitude for *Argyrosomus hololepidotus* populations.

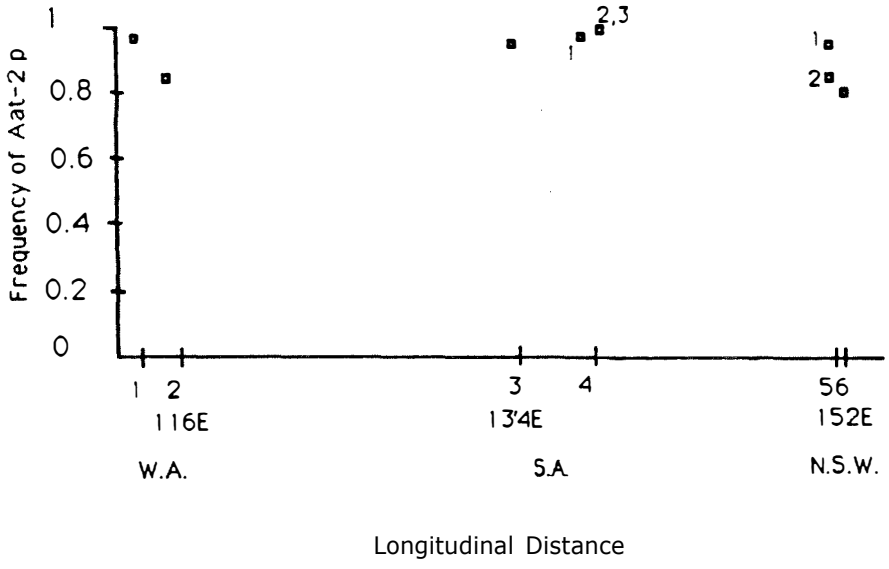


Figure 9 Scattergram of Aat-2 q-allele frequencies by longitude for *Argyrosomus hololepidotus* populations.

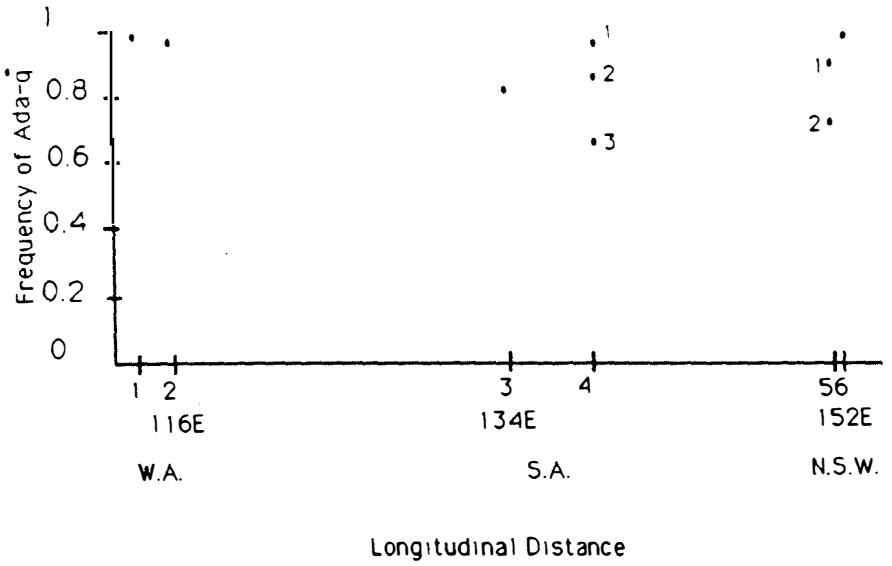


Figure 10 Scattergram of Ada q-allele frequencies by longitude for *Argyrosomus hololepidotus* populations.

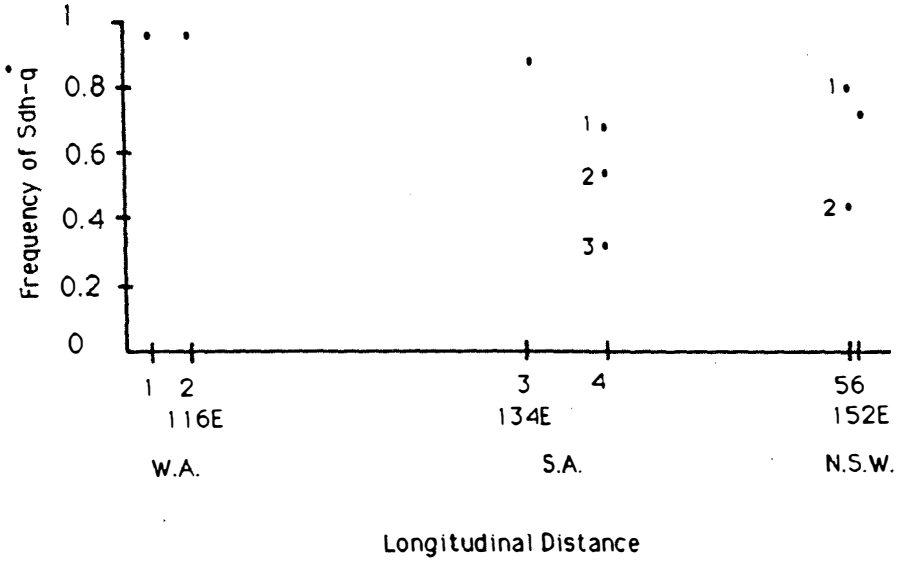


Figure 11 Scattergram of Sdh q-allele frequencies by longitude for *Argyrosomus hololepidotus* populations.

Table 3 Results of G-tests performed using all the allele frequency data.

		2	3	4	5	6	7	8	9
1	CARN/SHBAY	*	*	*	*	*	*	*	*
2	MAND		*	*	*	*	*	*	*
3	WESTSA			*	*	*		*	*
4	COOR1				*	*	*	*	*
5	COOR2					*	*	*	*
6	COOR3						*	*	*
7	HAWK1							*	*
8	HAWK2								*
9	HUNTER								

Table 4 Results of the genic contingency Chi-square test performed pooling allele frequency data from the S.A. and N.S.W. collections only.

Locus #	# alleles	$\sum$ N	chi^2	d.f	Prob.
1. Aat-2	2	461	62.733	6	*
2. Ada	2	525	123.477	6	*
3. Adh	3	630	40.271	12	*
4. Sdh	2	568	101.281	6	*

Table 5 Results of G-tests performed using the allele frequency data from Adh only.

		2	3	4	5	6	7	8	9
1	CARN/SHBAY	*	*	*	*	*	*	*	*
2	MAND		*	*	*	*	*	*	*
3	WESTSA								
4	COOR1						*		
5	COOR2					*	*		*
6	COOR3						*		
7	HAWK1								
8	HAWK2								
9	HUNTER								

Key: * indicates a significant result ($p<0.01$).

Table 6 Values of G for tests performed for the goodness-of-fit of allele frequencies to Hardy-Weinburg equilibrium.

	POPULATION	LOCUS			
		Aat-2	Ada	Adh	Sdh
1	CARN/SHBAY	-	-	10.6884*	-
2	MAND	0.1718	-	0.0749	-
3	WESTSA	-	0.8606	1.8559	1.4593
4	COOR1	-	-	8.5852*	8.5330*
5	COOR2	-	-	11.5001*	0.6434
6	COOR3	-	1.0309	0.6037	1.4987
7	HAWK1	2.8280	-	0.0818	6.0221*
8	HAWK2	2.6584	7.2204*	0.1686	0.3277
9	HUNTER	20.4624*	-	4.3842*	8.9051*

Key: * indicates a significant result, i.e. out of H-W, - indicates the cell was not tested as the frequency of the most common allele was >0.9. Degrees of Freedom = 1. Rare alleles pooled for the Adh locus.

Table 7 Numbers of samples not scored due to no staining reaction on the gel.

	POPULATION	LOCUS				TOTAL NO. OF
		Aat-2	Ada	Adh	Sdh	FISH SCREENED
1	CARN/SHBAY	34	9	0	13	44
2	MAND	4	0	0	3	30
3	WESTSA	3	22	5	4	36
4	COOR1	32	1	0	17	85
5	COOR2	92	43	1	2	136
6	COOR3	25	0	0	26	73
7	HAWK1	13	37	1	17	146
8	HAWK2	0	1	7	10	44
9	HUNTER	21	14	1	2	125
	TOTAL	224	127	15	94	719
	% OF SAMPLE	31%	18%	2%	13%	

Nei's genetic distance, D* (as modified by Hillis, 1984) was calculated from the allele frequency data using the program NEIBOTH. These data (see Appendix 4, Table 4.3) were put into the program FITCH (Felsenstein, 1981, 1982) to construct a phylogenetic tree. The dendrogram, shown in Figure 3, is presented here because of the widespread application of these measures in electrophoretic studies. The groupings on this dendrogram are chaotic, and the separation of the Western Australian samples (shown in the CONTML dendrogram) is lost using distance data.

The allele frequency data and numbers file (see Appendix 4, Tables 4.1 and 4.2) were input into the program POPSEP to test for differences between all possible pairs of populations. The G-tests showed significant differences between and within all the sampling sites (see Table 3), with the exception of the first collection in Hawkesbury River, N.S.W. and the collection from the west coast of S.A. Thus the possibility of further population sub-structuring between the Coorong and N.S.W. cannot be discounted. The differences observed between the W.A. samples may not be a true indication of substructuring occurring between these two sites as the sample numbers from both are small (see Appendix 4, Table4.2).

The allele frequency data and numbers file (see Appendix 4, Tables 4.1 and 4.2) were put into the program WEIGHTED CHI-SQUARE to test for population variance at each locus for the pooled collections made in S.A. and N.S.W.. The results, shown in Table 5, show significant population heterogeneity across all the loci examined.

The G-test was again performed for the Adh locus only, as this locus showed clearly (Figures 4 and 8a) the separation between the Western Australian fish and the eastern sub-population. Table 5 shows significant differences between both W.A. collections and all other eastern states, but no difference within the two W.A. sites. The differences between the first collection in the Hawkesbury River, N.S.W. and all the Coorong collections are significant, again indicating that there could be some genetic isolation between the fish collected in these two areas.

The nature of the within-site heterogeneity needs to be examined further before any conclusions can be drawn from these between-site differences. At both localities where more than one collection was made over subsequent years we have differences as large as those found between each locality, and furthermore, we find an increasing proportion of rarer alleles over time. Allele frequencies where the proportion of the most common allele is less than 0.9 were examined for goodness-of-fit to Hardy-Weinburg Equilibrium. Ten (out of 23 tests performed) did not fit Hardy-Weinburg expectations (see Table 6), showing significant heterozygote deficit in all cases.

Table 8 Values of Smith's H calculated from the allele frequency data showing significant deviations from Hardy-Weinburg equilibrium.

	POPULATION	LOCUS	SMITH'S H	95% CONFIDENCE INTERVAL
1	CARN/SHBAY	Adh	0.0999	0.0017
2	COOR1	Adh	0.0761	0.0013
		Sdh	0.0836	0.0015
3	COOR2	Adh	0.0675	0.0007
4	HAWK1	Sdh	0.0388	0.0004
5	HAWK2	Ada	0.0883	0.0019
6	HUNTER	Aat-2	0.0776	0.0005
		Adh	0.0446	0.0008
		Sdh	0.0606	0.0090

Possible explanations for these results may be:-

1/ scoring errors in assigning genotypes,

2/ genetic drift,

3/ population substructuring with either:

(a) inbreeding,

or, (b) overlapping sub-populations with different breeding cycles.

Scoring errors may have occurred due to the rapid deterioration of our tissue samples, where a significant number of specimens that did not stain may have in actuality been heterozygotes of weaker band strength (Richardson *et al*, 1986). Table 7 shows the numbers of specimens for each locus that were not scored due to no staining activity on the gel. As this table shows, we had major problems with poor sample quality for 3 of the 4 loci studied, where enzymes from more than 5% of fish did not stain. Of the 10 cells found to be out of H-W equilibrium, 4 have numbers of non-staining specimens large enough to affect H-W calculations if most of these were in actual fact heterozygotes. Those affected were 2 (out of 4 tests) for the Aat-2 locus, and 2 (out of 7 tests) for the Sdh locus. We therefore conclude that, although scoring errors may be an added complicating factor in our data, they alone cannot explain all the inter-locality heterogeneity observed in mulloway.

Genetic drift may occur if a large population is dramatically reduced, but there is no evidence of this being a recent occurrence with mulloway populations.

The last alternative is population substructuring, with either (a) inbreeding, or (b) overlapping sub-populations with different breeding cycles. Table 8 shows the

Smith's H values calculated for the 6 populations showing significant deviations from H-W. All values of H are positive, and the confidence intervals do not include zero, so our data shows significant heterozygote deficit (Wahlund effect). If mulloway in S.A. and N.S.W. consist of two wide-ranging, but discrete sub-populations, then selective harvesting is possible (Richardson *et al*, 1986). Overfishing of one sub-population (and possibly, underfishing of the other) could lead to the collapse of one sub-population within the fishery.

Discussion

Much of our data is not statistically reliable due to small sample size, both in the initial collections (4 of the 9 collections made) and the subsequent reduction due to the significant levels of no staining activity on the gels. However, overall trends can be seen.

We have evidence for a separate sub-population in Western Australia, with a discontinuity in allele frequencies at the Adh locus, and to a lesser extent, the Sdh locus. However, further data needs to be collected to determine the exact nature of this discontinuity. Tag returns will give essential information on the extent of mixing (if any) occurring between these geographic areas, and further collections of fish are required at suitable localities between S.A. and W.A. for electrophoretic screening before this discontinuity can be mapped.

No conclusions may be drawn about population structure in Western Australian waters due to the small numbers of fish available from the two localities sampled.

We examined mulloway from S.A. through to N.S.W., to test whether these fish belong to a single sub-population. The collection from the Hunter River, N.S.W. (made over a 3 week period in November and December), and repeated collections (over a time span of months to years) in the Coorong, S.A. and the Hawkesbury River, N.S.W. show significant inter-locality heterogeneity at two loci (Sdh and Adh). Furthermore, an increasing proportion of rarer alleles was observed for both the N.S.W. and S.A. sites. These results indicate that there may be more complex population sub-structuring occurring. However, genetic data alone is insufficient for any conclusions to be made, and other biological information (such as gonodosomatic index and other breeding information, and tagging data) is essential to determine the nature of this inter-locality heterogeneity.

Isoelectric focusing (IEF)

In mulloway, small differences were detected in the minor bands between each of the areas represented. Three patterns showing minor differences were found in the Hawkesbury River samples (see Figure 9), the Macleay River samples showed 3 patterns, and 2 patterns in the Shark Bay specimens (see Figure 10). Some differences within the major protein bands were revealed between each of the species investigated. In the *Austronibea oedogenys* samples, each of the 5 specimens produced a different pattern of bands. Four patterns had differences in the minor bands only. However, 1 pattern showed a difference in a major band, indicating the misidentification of this specimen (See Figure 10).

The gels were scored for presence or absence of a band in those regions where comparison across all samples was possible, firstly in the mulloway from the various collection sites represented, and secondly to compare mulloway to other Sciaenid species. Each individual sample was compared and classed into like groups. The character state data input to Felsenstein's MIX program is given in Appendix 4, Table 4.5. The dendrogram presented in Figure 14 is the last of 85 equally parsimonius Wagner phylogenetic trees from the MIX output.

In mulloway, there are 5 major groupings, from 6 localities around Australia. However, the clustering of localities is chaotic. Note that the W.A. sub-population separation of mulloway that was indicated by the allozyme data was not detected here.

For the other Sciaenid species examined, two specimens of *Austronibea oedogenys* clustered within the *Johnius vogleri* group. The misidentification of specimen No. 2 was expected from the description of the banding patterns, above. However, the grouping of specimen No.3 was not expected. This abberant result is most likely due to the loss of information on band strength when classifying for presence or absence of bands.

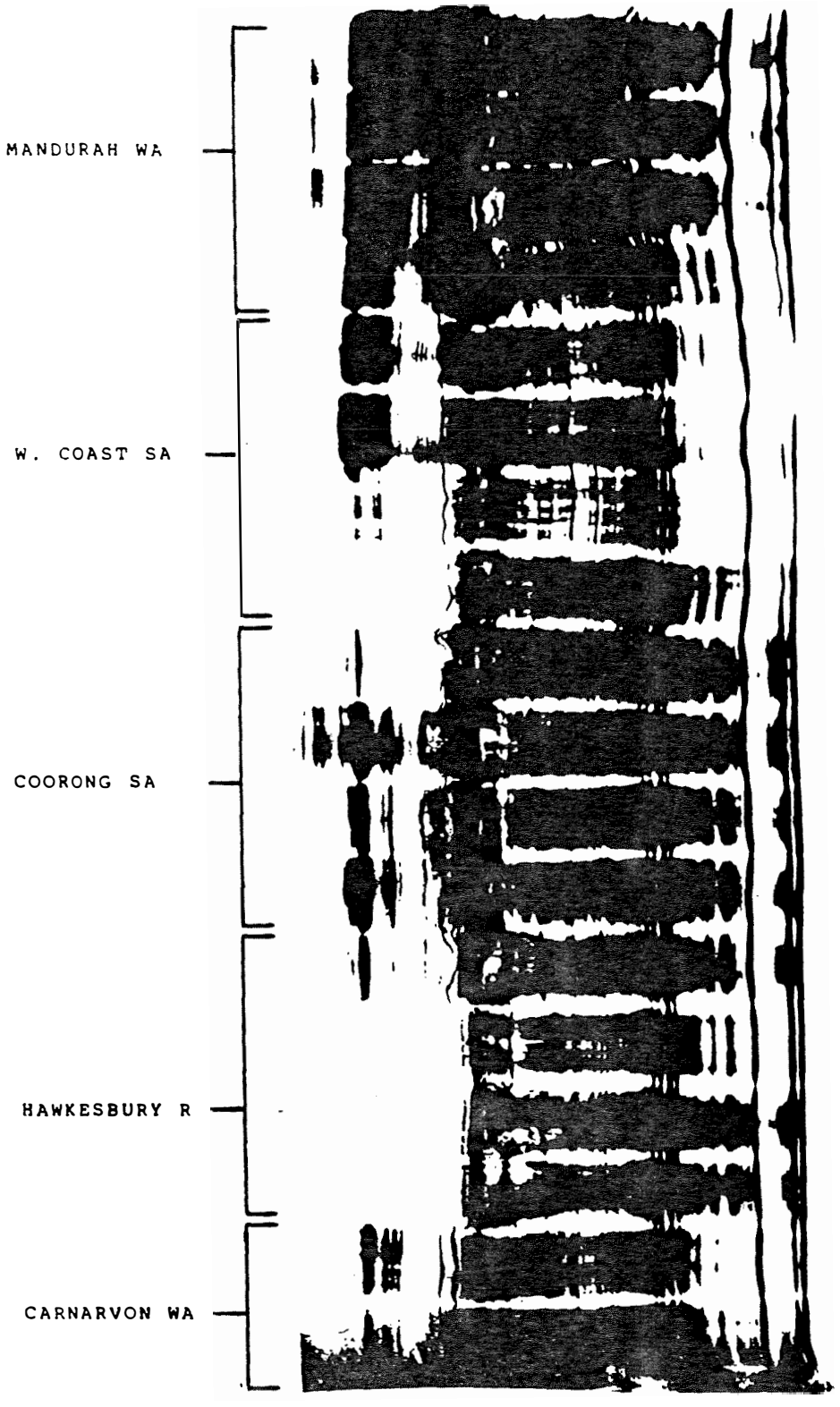


Figure 12 Isoelectric focusing gel for *Argyrosomus hololepidotus* (IEF pH 3.5-9.5, Page blue stain).

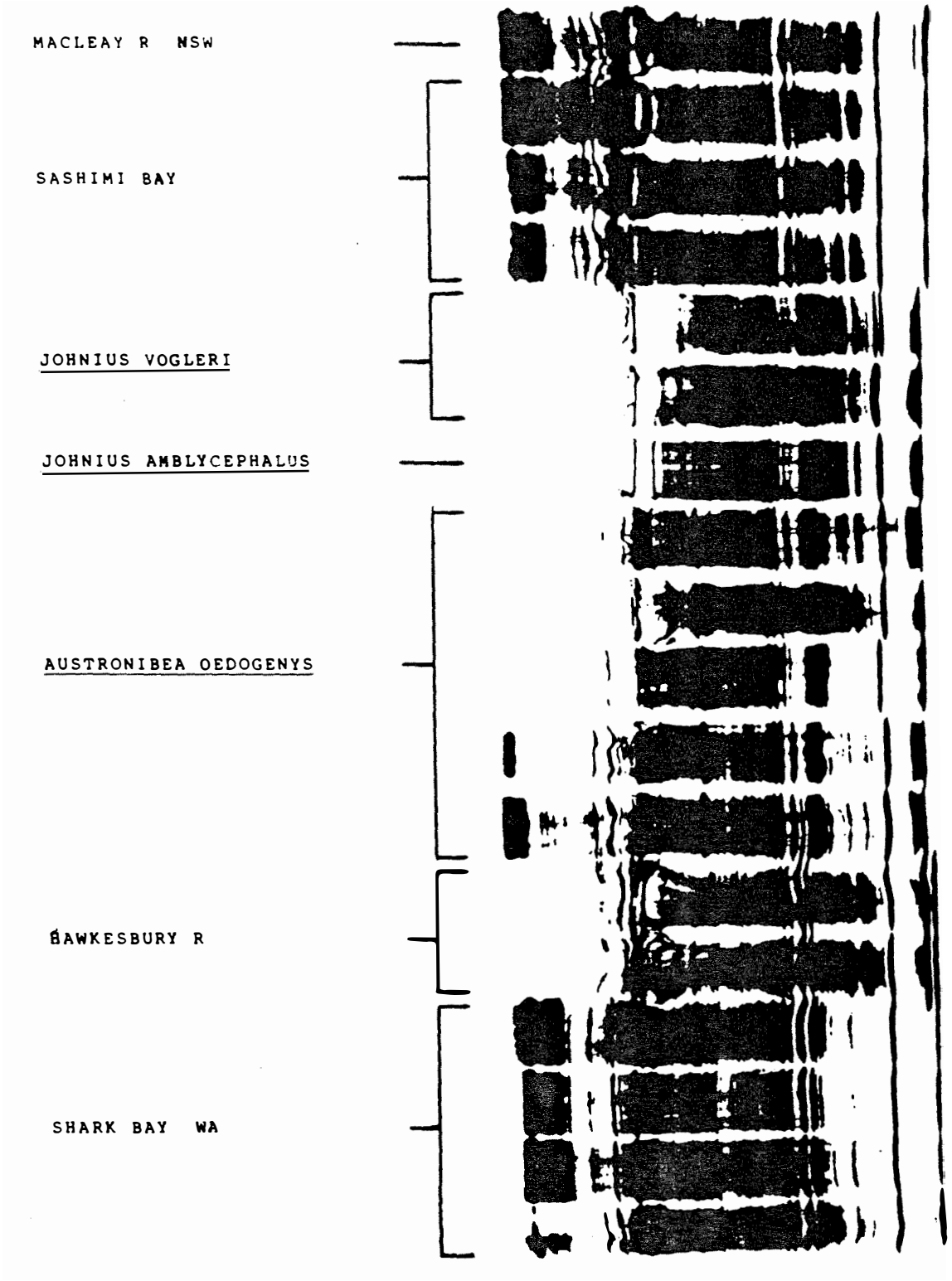
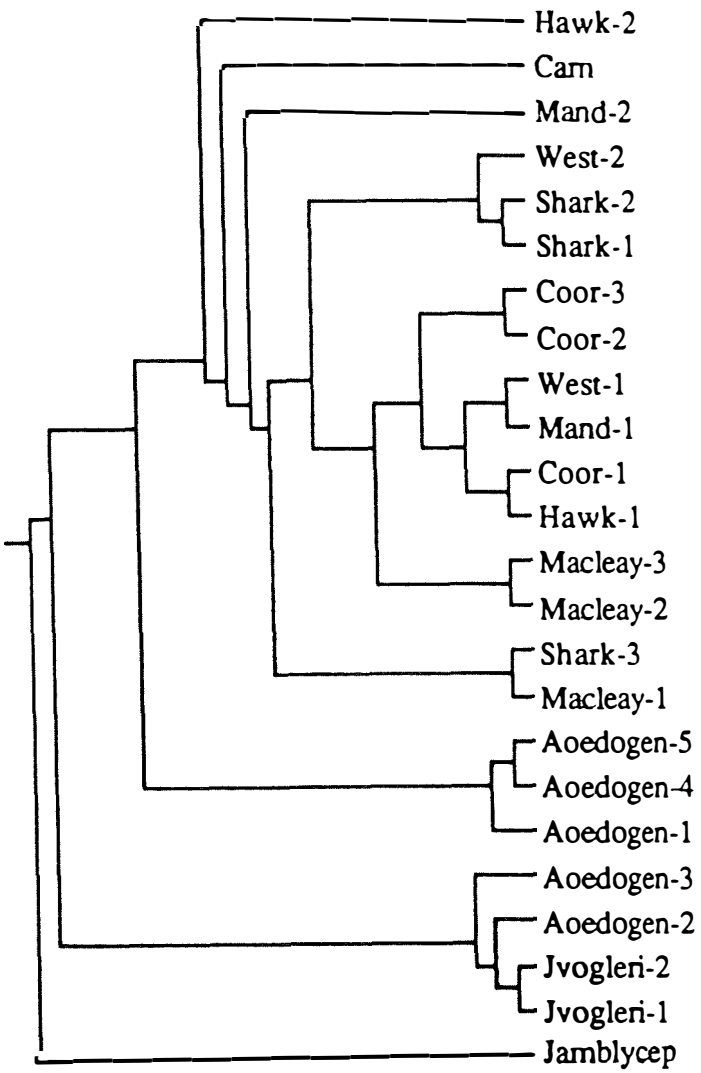


Figure 13 Isoelectric focusing gel for Sciaenid species (IEF pH 3.5-9.5, Page Blue stain).



Key: Jamblycep = *Johnius amblycephalus* No. 20; Aoedogen-1 to 5 = *Austronibea oedogenys* Nos 21-25; Jvogleri-1 and 2 = *Johnius vogleri* Nos 18 and 19; *Argyrosomus hololepidotus* : Macleay = Macleay River, NSW. 1 = Nos 26, 28, and Shark Bay, WA. No. 14, 2 = No. 27, 3 = No. 29; Shark = Shark Bay, WA. 1 = No. 11, 2 = Nos 12 and 13; Hawk = Hawkesbury River,NSW. 1= No. 143, 2= Nos 142 and 144; Mand = Mandurah, WA. 1= Nos 26-29, Hawk 141, and West 26, 2 = No. 28; West = West Coast, SA. 1 = Nos 27 and 28, 2 = No.29; Coor = Coorong, SA. 1 = Nos 131 and 133, 2 = No. 132, 3 = No. 134; Carn = Carnarvon, WA. 1 = No. 25, 2 = No. 24.

Figure 14 Wagner phylogenetic tree for Sciaenid species (MIX output).

Mitochondrial DNA (MtDNA)

Restriction digest fragments of mtDNA

The rapid technique for the isolation of mtDNA from fish, published by Chapman and Powers (1984), was initially investigated under the guidance of Dr J. Ovenden at the University of Tasmania. We used 5 liver samples from:- the west coast, 2 individuals; Coorong, 2 individuals; Hawkesbury River, 1 individual; and 1 female gonad at stage 2 from the Coorong. Only the gonad sample showed a small amount of supercoiled mtDNA on agarose gel when visualised with ethidium bromide. All samples were contaminated with sheared DNA, producing prominent background smears. The restriction enzymes investigated to cut this material were Ava-I (having a 4 base recognition sequence), Bgl-I, Pvu-II, and Xho-I (all having 6 base recognition sequences). Only Ava-I digested the DNA, revealing 4 very faint bands, the largest of which was only in the order of 4000 base pairs (bp) (see Figure 15). A HindIII digest, followed by end-labelling for visualisation, was conducted by Dr Jenny Ovenden on the six mtDNA preparations undertaken in that laboratory. Against high background contamination, 3 bands were revealed in 3 liver samples and the single gonad sample, again in the order of 4000bp or less (see Figure 16).

The size of mtDNA of most animals and fish has been determined to be in the order of 16,000 to 17,000 base pairs. (Figure 17 shows mulloway mtDNA to be >16000bp standard). We expect from a restriction digest of intact mtDNA fragment sizes adding to approximately this range. However, from mulloway the largest fragment is in the order of 4000bp. Adding the 2 or 3 smaller fragments does not approximate the expected size of the mitochondrial genome. This result indicates that the mtDNA obtained has been sheared into smaller fragments by extraneous factors, either before or during the restriction enzyme digest.

Slightly better results were obtained at U.N.S.W. We used 2 liver and 2 female gonad samples from fish collected from Port Stephens and Seal Rocks, N.S.W. Serial dilutions were performed to check the yield (see Figure 17). The minigel shows a mtDNA band in each sample at a position in the gel>16,000bp (from the standard). However, yields were sufficient for no more than two restriction digests, at best. Smearing of contaminating DNA is a greater problem in the liver samples. The Port Stephens gonad sample shows two extra bands at <16,000bp, indicating that some of the mtDNA has been cut (before any digests have been performed). The Seal Rocks samples are very faint, again indicating that tissue sample collection conditions are critical for mulloway. No cuts were revealed from digests using EcoR1 and Pst-1.

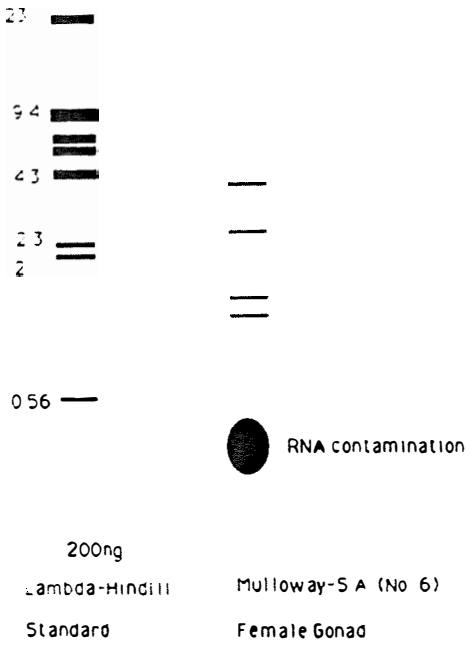


Figure 15 Diagramatic representation of restriction digest of mtDNA from female gonad using Ava-1. (1% agarose/ethidium bromide gel in TPE, 60V, 1h).

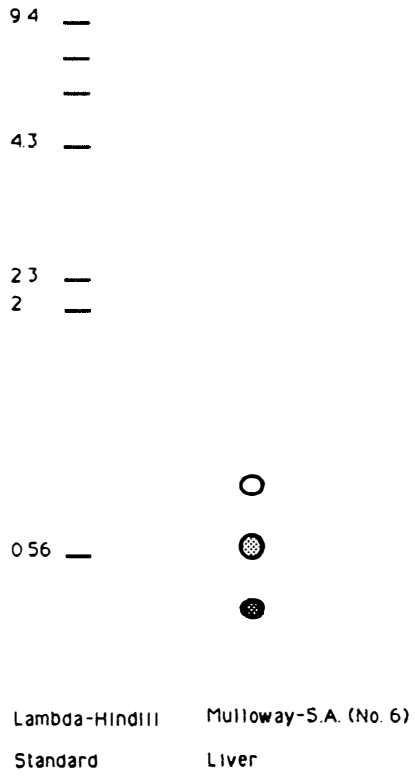


Figure 16 Diagramatic representation of restriction digest of mtDNA from five liver samples and one female gonad using HindIII. (end-labelled with ^{32}P dCTP, 1.4% agarose gel in TPE, overnight exposure).

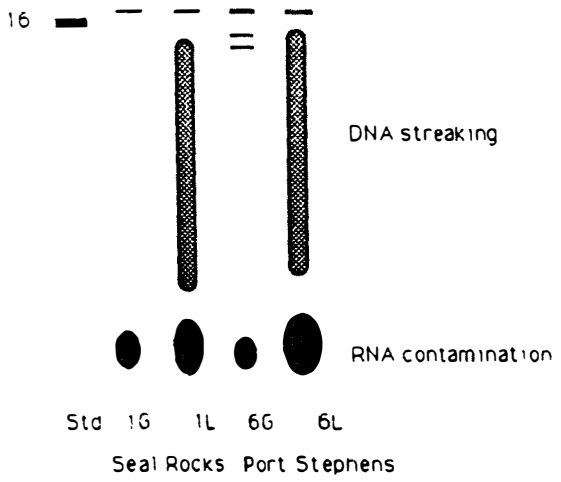


Figure 17 Miniprep showing yield of mtDNA from liver and gonad tissue. Chapman and Power's prep. (0.8% agarose/ethidium bromide gel in TAE, 60V, 1h).

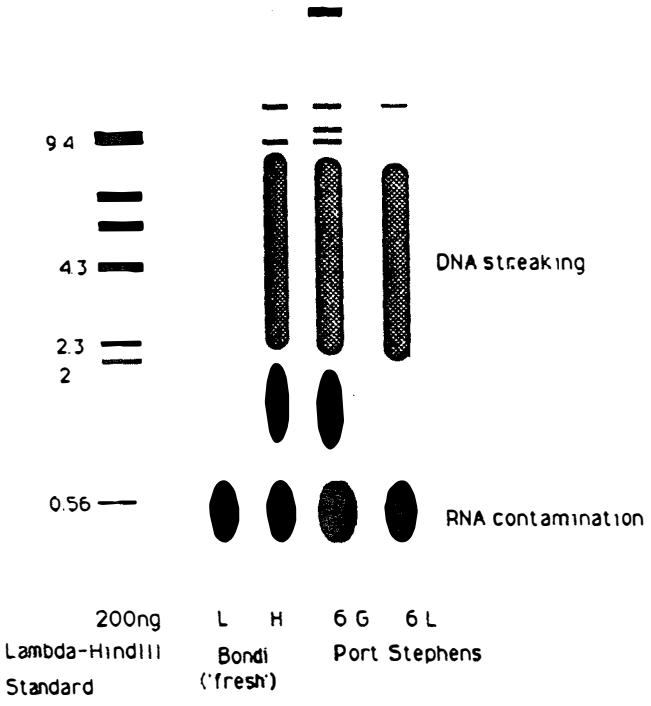


Figure 18 Miniprep showing yield of mtDNA from fresh liver, heart and gonad tissue. Lansman prep. (0.8% agarose/ethidium bromide gel in TAE, 60V, 1h).

As we required a cleaner preparation of mtDNA and a greater yield, the more thorough technique utilising sucrose step gradient and $CsCl_2$ density gradient ultracentrifugation, published by Lansman *et al.* (1981), was investigated. We used 2 liver samples from the one Terrigal fish. No mtDNA was recovered from the second $CsCl_2$ density gradient. A modification, following this method to the sucrose step gradient and precipitating the mitochondrial lysis at this stage, was investigated using 2 liver, 1 heart, and 2 female gonad samples from individuals from Port Stephens and Bondi, N.S.W. The tissues from the Bondi fish were not previously frozen. The heart and gonad samples from this fish revealed small amounts of highly contaminated mtDNA (see Figure 18). Yields obtained from this more rigorous method are only a slight improvement from those obtained previously from the rapid method (see Figure 16). No bands were revealed from digests using BamHI and Pst-I1.

Appendix 6 gives details of the restriction enzymes used and the digest protocol followed for this study. Of a total of 9 restriction enzymes screened, we found 2 that have recognition sequences in mulloway, namely Ava-1 and HindIII.

We previously suggested that any future investigations should be directed towards obtaining a clean preparation of mtDNA using large amounts of fresh heart and maturing eggs from a few fish by following the longer method of $CsCl_2$ density gradient ultracentrifugation. If enough mtDNA could be obtained, and labelled with the non-radioactive digoxigenin-dUTP, it could then be used as a probe to detect mtDNA from the frozen liver or gonad samples. A crude extract of total DNA (prepared from the samples stored cryogenically) is digested with selected restriction enzymes. Any mtDNA fragments may then be visualized from a southern blot of the agarose gel using the probe.

Mulloway continued to prove difficult, both in terms of obtaining fresh material at an appropriate time for laboratory work to proceed, and in the yield of supercoiled mtDNA. It may be possible to utilize mtDNA from a closely related species to probe for restriction digest fragments, although recent discussion with Dr. J. Avise (University of Georgia, USA) confirmed that mtDNA of Sciaenids is very difficult to work with. With this in mind, no further work with restriction enzymes was undertaken. Instead we turned to the alternative approach of direct sequencing that is proving useful for many species.

Sequencing mtDNA

Two regions were selected for sequencing:-the cytochrome b gene and the D-loop. Nucleotide sequence evolution within mitochondrial DNA occurs at different rates in different regions of the DNA. The cytochrome b gene is a protein coding gene and as such has more evolutionary constraints than the D-loop region, much of which is non-coding. More sequence variation therefore is to be expected in the D-loop region than in the cytochrome b gene.

(a) Sequence of the cytochrome b region

Sequence data was obtained for 5 mulloway from the Hawkesbury River. The number of nucleotides sequenced were as follows:

Sample 1	162bp's
Sample 2	114bp's
Sample 3	208bp's
Sample 4	140bp's
Sample 5	257bp's

No sequence variation was found between these samples. Comparison of the Hawkesbury sequence with sequences from the Coorong, W.A. (1 individual, 218 base pairs), west coast of S.A. (1 individual, 120 base pairs) and Bondi, NSW (1 individual, 71 base pairs) revealed no variation. The partial sequence of the cytochrome b region for the seven sample sites is shown in Figure 16.

A sequence closer than approximately 20 base pairs to either primer was unobtainable. The shortness of some sequences was due purely to the time constraint and not any technical problems. The Carnarvon samples, however, did prove hard to sequence and only small stretches of unambiguous sequence were obtained. These samples were initailly collected for allozyme electrophoresis, and hence were not subject to the more stringent conditions imposed for mtDNA sampling. As the cytochrome b gene consists of 310 base pairs, it is possible that the remaining nucleotides (approximately 1/3 of the gene) may contain interpopulation variation.

(b) D-Loop fails to amplify

Amplification of the D-loop region was unsuccessful. Even when annealing and extension temperatures were lowered to the less stringent 46^0C and 68^0C respectively, no product was observed, indicating a large amount of primer-template mismatch. This is not altogether surprising given the rapid rate of evolution in this region.

```
WCSA          AAACTTTGCTCTCTGCTCGGCCCTTTGCTTAGCCGCCCAAATTCTCACACGACTCTTCCT
CRONULLA                    ........................................
HAWKES R.                    .......................................
BONDI
COORONG                                          ......................
CARNARVON
TERRIGAL

WCSA          TGCTATACACTACACATCCGACATCTCCATGGCCTTCTCATCCGTCGCACACATTTGCCG
CRONULLA      ............................................................
HAWKES. R.    ............................................................
BONDI                ......................................................
COORONG       ............................................................
CARNARVON
TERRIGAL              .....................................................

WCSA          AGATGTTAACTACGGATGGCTCATCCGAAACCTCCAGCCCAACGGCGCCTCTTTCTTCTT
CRONULLA      ............................................................
HAWKES. R.    ............................................................
BONDI         ............................................................
COORONG       ............................................................
CARNARVON                                  .....       ......    ......
TERRIGAL      ............................................................

WCSA          TATCTGCCTTTACCTCCACATCGGCCGAGGCCTCTACTATGGCTCTTATCTTTATAAAGA
CRONULLA      ............................................................
HAWKES. R.    ............................................................
BONDI         ............................................................
COORONG       ............................................................
CARNARVON     ..............      ............
TERRIGAL      ............................................................

WCSA          AACATGAAACATTGGAGTCGTACTCTTCCTTTTAGTAAT
CRONULLA      ....................................AATGACTGCC
HAWKES. R.    ................................................TTCGTAGGCTA
BONDI         ............................................................
COORONG       ............................................................
CARNARVON
TERRIGAL      ..........................................................
```

Figure 19 Partial sequence for the cytochrome b region for seven sample sites. Gaps indicate regions of unobtained sequence (or in the case of the Carnarvon samples regions of ambiguous sequence). Dots indicate shared nucleotides. No nucleotide variation was found. Abbreviations are as follows: WCSA- West Coast South Australia, Hawkes. R.- Hawkesbury River.

Discussion

The lack of nucleotide variation in the cytochrome b region suggests a single interbreeding population of mulloway stretching south from Sydney to at least the south west coast of South Australia. This is, however, by no means a certainty. A population phylogeny based on the sequence data from one locus will not necessarily be the same as the true phylogeny. This is due to a time-depth problem (Crozier, 1990). If speciation (or, as in this case, population isolation) is a recent event then differences in the sequences of the daughter populations may be the result of ancestral polymorphism in the parental population. The effects of such ancestral polymorphism may be overcome (or at least detected) by sampling more than one copy of the gene from each population. This was done for six of the seven sample sites and no variation was found. This may support the idea of a single population, but is in no way conclusive (especially considering the fact that only a relatively small number of nucleotides were sequenced). The best way to reduce the possible effects of ancestral polymorphism is to sample more than one locus.

The slow rate of evolution of the cytochrome b gene begs us to be wary of considering the data obtained as a true representation of the population structure. Crozier (1990) has questioned the ability of the more slowly evolving regions of mtDNA to discern differences between closely related populations or species. It is for this reason that the more rapidly evolving D-loop was considered for this study. The lack of data from this region is therefore very disappointing as it would appear to have more potential in elucidating population relationships. It should be noted however that the evolutionary rates of different regions of mtDNA are at best a rough guide in such analyses. The uncertainty as to the time separation between populations and the possibility of varying evolutionary rates between lineages affords us no guarantee as to which region would be best sequenced for which study.

The inability to retrieve an unambiguous sequence for the Carnarvon samples means that the possibility of two subpopulations hinted at by electrophoresis could not be addressed. Sequences obtained did appear in some instances to exhibit nucleotide variation, but in all cases bands appeared in more than one lane thus making correct resolution of the sequence impossible. It is strongly suggested that further sequencing of Carnarvon populations be undertaken to resolve this problem.

Further attempts at determining population stucture in mulloway would best be served by continuing attempts at sequencing the D-loop region of mtDNA. The failure of the primers used in this study should not rule out the role this non-coding region can play. Effort should now be directed at finding those primers that will amplify this region. This may involve cloning restriction fragments of the region to find conserved areas from which primers can be constructed. The wealth of sequence data that is now becoming available for this region (and mtDNA in general) may however make this process unnecessary. Other primers mentioned in the literature may indeed amplify the region that our primers could not. It is also recommended that a nuclear DNA locus be sequenced. Although nuclear loci alone are less likely than mtDNA loci to return the true tree due to their larger effective population size, they play a significant role in resolving time-depth problems (Crozier, 1990).

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APPENDIX 1 Details of enzymes surveyed for genetic variation in *Argyrosomus hololepidotus*..

Enzyme	Abbreviation	Enzyme Commission Number
Acid phosphatase	Acph	EC 3.1.3.2
Aconitase	Acon	EC 4.2.1.3
Adenosine deaminase	Ada	EC 3.5.4.4
Adenylate kinase	Ak	EC 2.7.4.3
Alcohol dehydrogenase	Adh	EC 1.1.1.1
Aldolase	Ald	EC 4.1.2.13
Alkaline phosphatase	Alkph	EC 3.1.3.1
Aspartate aminotransferase	Aat	EC 2.6.1.1
Carbonic anhydrase	Ca	EC 4.2.1.1
Catalase	Cat	EC 1.11.1.6
Diaphorase	Dia	EC 1.6.2.2
D-aminoacid oxidase	Damox	EC 1.4.3.3
D-aspartate oxidase	Dasox	EC 1.4.3.1
Esterase	Est	EC 3.1.1.1
Fumarase	Fum	EC 4.2.1.2
Glyceraldehyde-3-phosphate dehydrogenase	Ga3pdh	EC 1.2.1.12
Glucose-6-phosphate dehydrogenase	G6pdh	EC 1.1.1.49
Glucosephosphate isomerase	Gpi	EC 5.3.1.9
Glutamate-pyruvate transaminase	Gpt	EC 2.6.1.2
alpha-glycerophosphate dehydrogenase	Gpd	EC 1.1.1.8
Guanine deaminase	Gda	EC 3.5.4.3
Hexosaminidase	Hex	EC 3.2.1.30
Hexokinase	Hk	EC 2.7.1.1
Isocitrate dehydrogenase	Idh	EC 1.1.1.42
Lactate dehydrogenase	Ldh	EC 1.1.1.27
Leucine aminopeptidase	Lap	EC 3.4.11.1

APPENDIX 1 cont...

Enzyme	Abbreviation	Enzyme Commission Number
Malate dehydrogenase	Mdh	EC 1.1.1.37
Malic enzyme	Me	EC 1.1.1.40
Mannosephosphate isomerase	Mpi	EC 5.3.1.8
Peptidases (FP,LGG,LLL,LY,PL,VL)	Pep	EC 3.4.11 or 13
Phosphoglycerate kinase	Pgk	EC 2.7.2.3
Phosphoglucomutase	Pgm	EC 5.4.2.2
Pyruvate kinase	Pk	EC 2.7.1.40
6-Phosphogluconate dehydrogenase	Pgd	EC 1.1.1.44
Sorbitol dehydrogenase	Sdh	EC 1.1.1.14
Superoxide dismutase	Sod	EC 1.15.1.1
Xanthine dehydrogenase	Xdh	EC 1.1.1.204

A total of 40 enzyme systems investigated.

APPENDIX 2 Enzymes studied, tissues investigated, electrophoresis running conditions and presumed number of loci for *Argyrosomus hololepidotus*.

Key: **L** = liver, **M** = muscle, **K** = kidney, **H** = heart
b = extraction buffer, **w** = deionised, distilled water
1 = Poulik, **2** = Tris-maleate pH 7.8, **3** = Citric acid-aminopropyl-morpholine pH 6.1
a. = anodal migration, **c.** = cathodal migration
P = polymorphic, **M** = monomorphic, **NS** = no staining activity
* = best tissue or buffer

Enzyme	Tissue	Buffer	Presumed no. of loci	Comments
Acph	L	3	1a	?P, try on cellogel-No
	K	1,2*	2a	streaks a
	H	3	2a	Acph-1 ?P check Alkph-No
Acon	L	1	1a	poor activity, M
	M	1	NS	
Ada	L	1	1a	M
	M	1,2*,3	1a	P, poor activity
Ak	L	3	2a	good activity
	M	3	2a	in all tissues
	H	3	1a	M
Adh	L	1,3*	1a	good activity and
	K	1	NS	resolution, P
Ald	L	1	1a	poor activity
	M	1*,2,3	1a	poor activity
Alkph	L	1*,2	1a	?P, streaks a
	K	1,2*,3	1a	?P, poor activity

APPENDIX 2 cont...

Aat	L	1,3*	1a,1c	poor resolution, Aat-1
	M^b	1,2,3*	1a,1c	good activity, warping
	K	1	2a	streaks a
	H	1,3*	1a,1c	Aat-2 P
Ca	H	1	1a	M, good activty
Cat	L	1	1a	good activity, but
	M	1	1a	poor resolution
Ck	L	2	1a	poor activity
	K^w	3	1a	M
	H	2	1a	warping
Damox	K	3	1a	on origin
Dasox	K	3	NS	
Dia	K^w	2	2a	no activity SA
Est	L	1	3a	Est-2 ?P
	M	1	3a	? NSW/WA difference
	K	1	1a	M
	H	1	2a	warping
Fum	L	2	NS	
Ga3pdh	M^w	1,2,3*	1a,1c	poor resolution
G6pdh	K^w	3	1a	poor activity
Gpi	L	1	1a	good activity, M
	M	1	2a	Gpi-1,2 P
	H	1	2a	Gpi-1,Gpi-2 P

APPENDIX 2 cont...

Enzyme	Tissue	Buffer(s)	Presumed no. of loci	Comments
Gpt	L	1	1a	poor resolution
	H	1	-	
Gpd	L	1,2*,3	1a	poor activity
	M	2	2a	?P, ?NSW/WA no too faint
	H	1	1	activity on origin
Gda	M	2	NS	
Hex	L	1,2*	1c	streaks c
Hk	K	2	NS	
Idh	L^w	1,3*	1a	? P
	M^w	2,3*	2a	?P, best tissue
	K^w	3	1a	?P
	H	3	2a	
Ldh	L	1	3a	sub-bands
	M	1	1a	M
	K	3	3a	good activity
	H	1	1a	M
Lap	L	2	1a	poor resolution
	K^b	2	1a	?P, poor activity
Mdh	L	1*,3	1a	good activity
	M	2,3*	2a	?P, ?NSW/SA difference
	K	3	1a	good activity
	H	2	1a	good activity

APPENDIX 2 cont...

Enzyme	Tissue	Buffer(s)	Presumed no. of loci	Comments
Me	L^W	1	1a	poor activity
	M^W	2	2a	Me-1 ?P, poor activity
	K^W	3	1a	poor activity
	H	2	1a	sub-bands
Mpi	L	1	1a	P
	M^W	1,3*	1a	P
	K^W	3	1a	variable
PepA (VL)	L	1	1a	M
	M	1	1a	M
	K	2	1a	M
PepB (LGG)	L	1	2a	P
	M	1	2a	P
	K	2	1a	
	H	2	1a	
PepC (PL)	K	2	1a	P, ?=PepB-1
PepD (FP)	L	1	1a	M
	M	1	1a	M
	K	2	1a	M
PepS (LLL)	L	1	NS	
	M	1	NS	
PepS(LY)	L	1	1a	M
	M	1	1a	
Pgk	L	1	2a	poor activity
	M	1	1a	warping
	H	1	NS	

APPENDIX 2 cont...

Enzyme	Tissue	Buffer(s)	Presumed no. of loci	Comments
Pgm	L^w	1	1a	poor activity
	M^w	1*,2,3	1a	P
	K	1	1a	not all samples stain
	H	1	2a	Pgm-1 P, ?NSW/WA
Pk	L	1	2a	Pk-1 good activity
	H	1	1a	under Hb band
Pgd	L	2	1a	warping
	K	1	NS	
Sdh	L^b	1*,3	1a	?P, sub-bands
	K^b	1	1a	?P, complex pattern
	H	1	NS	
Sod	L	1	1a	M
	K	2	1a	M
Xdh	L^w	1*,3	1a	warping
	K^w	1	NS	
	H	1	1a	poor activity

A total of 40 enzyme systems investigated, 37 showed activity, representing 56 presumptive loci.

Appendix 3 Description of enzyme banding patterns for all the polymorphic loci selected for screening collections of *Argyrosomus hololepidotus*.

Alcohol dehydrogenase (ADH) EC1.1.1.1

ADH was examined in extracts of liver tissue. A single locus migrates anodally in Poulik buffer.

Subunit structure : dimeric

Banding Pattern : single band in monomorphic fish, heterozygotes show 3 bands Three alleles were detected, "b" common. Figure 3.1 is a diagramatic representation of the observed phenotypes.

Adenosine deaminase (ADA) EC 3.5.4.4

ADA was examined in extracts of muscle tissue. A single locus migrates anodally in TM pH 7.8 buffer.

Subunit structure : monomeric

Banding Pattern : single band in monomorphic fish, heterozygotes show 2 bands. Three alleles were detected, "b" common. Although this locus ocassionally showed poor intensity of activity in mulloway, and many samples did not stain, we were able to use this locus in our analyses. Figure 3.2 is a diagramatic representation of the observed phenotypes.

Aspartate aminotransferase (AAT) EC 2.6.1.1

AAT was examined in extracts of heart or muscle tissue. Two loci are present - Aat-1 is monomorphic and migrates cathodally, Aat-2 is polymorphic and migrates anodally in CAM pH 6.1 buffer.

Subunit structure : dimeric

Banding Pattern : single band in monomorphic fish, heterozygotes show a larger staining zone. Two alleles were detected, "a" common. However, this locus is prone to warping, and showed poor staining intensity in mulloway with many samples not staining. Therefore, this locus was only able to be used to compare selected population sets. Figure 3.3 is a diagramatic representation of the observed phenotypes.

x

APPENDIX 3 cont...

Esterase (EST) EC 3.1.1.1

EST was examined in extracts of heart tissue. Two (or three) loci migrate anodally in CAM pH 6.1 buffer.

Subunit structure : monomeric or dimeric

Banding Pattern : The least anodally-migrating locus showed poor staining intensity in mulloway. As the banding pattern was difficult to interpret, EST was not included in the analyses. The possibility of a null allele is indicated, with the absence of any band. Two loci may be indicated with one, two or sometimes three bands staining. The most anodally-migrating locus showed strong staining activity, however it is monomorphic. Figure 3.4 is a diagramatic representation of the observed banding patterns.

Glucosephosphate isomerase (GPI) EC 5.3.1.9

GPI was examined in extracts of muscle tissue. Two loci migrate anodally in TM pH 7.8 buffer. Both loci are polymorphic.

Subunit structure : dimeric

Banding Pattern : single band in monomorphic fish, heterozygotes show 3 bands. Two alleles were detected, "a" common for both loci. As the frequency of heterozygotes was very low (a total of 6 out of 720 fish screened), GPI was not included in the final analyses. Figure 3.5 is a diagramatic representation of the observed phenotypes. Note the presence of hybrid heteropolymer bands intermediate to Gpi-1 and Gpi-2.

Isocitrate dehydrogenasease (IDH) EC 1.1.1.42

IDH was examined in extracts of heart tissue. Two loci migrate anodally in CAM pH 6.1 buffer.

Subunit structure : dimeric

Banding Pattern : single band in monomorphic fish, heterozygotes were atypical in showing 2 bands of activity, or bands merge into a larger staining zone. 2 alleles were detected, "a" common for Idh-1, "b" common for Idh-2. However, this locus showed a variable intensity in activity for mulloway, and many samples did not stain, so it was not included in our analyses. Figure 3.6 is a diagramatic representation of the observed phenotypes.

APPENDIX 3 cont...

Malate dehydrogenase (MDH) EC 1.1.1.37

MDH was examined in extracts of muscle tissue. Two loci migrate anodally in TM pH7.8 buffer.

Subunit structure : dimeric

Banding Pattern : As the banding pattern was variable and difficult to interpret, MDH was not included in the analyses. We were unable to decipher between a single locus with anodal sub-bands, or 2 loci (one or both polymorphic) migrating to the same region of gel. This locus was not included in our analyses Figure 3.7 is a diagramatic representation of the observed phenotypes.

Mannosephosphate isomerase (MPI) EC 5.3.1.8

MPI was examined in extracts of liver tissue. A single locus migrates anodally in Poulik buffer.

Subunit structure : monomeric

Banding Pattern : single band in monomorphic fish, heterozygotes show 2 bands of activity, or bands merge into a larger staining zone. Two or three alleles were detected, "b" common. However, this locus is prone to warping and sub-banding, and many of the "standards" repeated on subsequent gels did not show the expected banding pattern. For these reasons MPI was not included in the final analyses. Figure 3.8 is a diagramatic representation of the observed phenotypes.

Peptidase (PEP) EC 3.4.11

PEPB was examined in extracts of liver tissue, using the tripeptide leucyl-glycyl-glycine (LGG) as substrate. Two loci migrate anodally in Poulik buffer. Both loci are polymorphic.

Subunit structure : monomeric

Banding Pattern : single band in monomorphic fish, heterozygotes show 2 bands of activity, or bands merge into a larger staining zone. Three alleles were detected in PepB-1, "a" common, however this locus is at times prone to poor resolution and warping, and was not included in our analyses. Three alleles were detected in PepB-2, "c" common. Figure 3.9 is a diagramatic representation of the observed phenotypes.

APPENDIX 3 cont...

PEPC was examined in extracts of liver tissue, using the dipeptide prolyl-leucine (PL) as substrate. A single locus migrates anodally in Poulik buffer.

Subunit structure : monomeric

Banding Pattern : single band in monomorphic fish, heterozygotes show 2 bands of activity, or bands merge into a larger staining zone. Two alleles were detected, "a" common, and stained like PepB-1. However, this locus showed poor intensity of activity in mulloway, and many samples did not stain, and we did not include this locus in our analyses. Figure 3.10 is a diagramatic representation of the observed phenotypes.

Phosphoglucomutase (PGM) EC 2.7.5.1

PGM was examined in extracts of muscle tissue. Two loci migrate anodally in TM pH7.8 buffer.

Subunit structure : monomeric

Banding Pattern : a single band in monomorphic fish, heterozygotes show 2 bands of activity. Pgm-1: 3 alleles were detected, "b" common. However, this locus showed great variation in intensity of activity in mulloway, from very active samples through to the many samples that did not stain. Pgm-2: is most active in liver and showed 2 alleles, "a" common. However, this locus also showed activity in muscle, so PGM was chosen to be screened in muscle tissue for convenience. The polymorphisms detected in the pilot screen of liver, however, were not reflected in subsequent screening of muscle tissue. As the frequency of heterozygotes was extremely low (a total of 2 out of 720 fish screened), PGM was not included in the final analyses. Figure 3.11 is a diagramatic representation of the observed phenotypes.

Sorbitol dehydrogenasease (SDH) EC 1.1.1.14

SDH was examined in extracts of liver tissue. A single locus migrates anodally in Poulik buffer.

Subunit structure : tetrameric

Banding Pattern : One or three bands in monomorphic fish, heterozygotes show 5 bands of activity, or bands merge into a larger staining zone. This locus is prone to smearing, sub-banding, and showed poor activity in many samples. Two alleles (a conservative estimate) were detected, "b" common. Figure 3.12 is a diagramatic representation of the observed phenotypes.

Appendix 4 Allele frequency and numbers input files for CONTML, POPSEP and NEIBOTH, genetic distance input file for FITCH, and character state input file for MIX.

Table 4.1 Allele frequency input file for CONTML, POPSEP and NEIBOTH.

9 Populations, 4 Loci

Numbers of alleles at the loci:

Aat-2	Ada	Adh	Sdh
2	2	3	2

Name	Phenotypes				
CARN/SHBAY	0.950	0.014	0.000	0.025	0.032
MAND	0.827	0.017	0.017	0.150	0.037
WESTSA	0.939	0.179	0.032	0.677	0.172
COOR1	0.981	0.030	0.012	0.624	0.353
COOR2	1.000	0.091	0.000	0.656	0.463
COOR3	1.000	0.329	0.000	0.603	0.681
HAWK1	0.929	0.079	0.062	0.510	0.215
HAWK2	0.841	0.279	0.014	0.635	0.574
HUNTER	0.803	0.023	0.036	0.625	0.321

Table 4.2 Numbers input file for POPSEP and NEIBOTH

	Aat	Ada	Adh	Sdh
CARN/SHBAY	10	35	44	31
MAND	26	30	30	27
WESTSA	33	14	31	32
COOR1	54	84	85	68
COOR2	44	93	135	134
COOR3	48	73	73	47
HAWK1	134	107	145	130
HAWK2	44	43	37	34
HUNTER	104	111	124	123

Table 4.3 Genetic distance input file for FITCH.

```
9 populations

Name                    Genetic distances
CARN/SHBAY 0.0000 0.0016 0.0003 0.0000 0.0000 0.0000 0.0018 0.0001 0.0004
MAND       0.0016 0.0000 0.0005 0.0011 0.0016 0.0016 0.0000 0.0010 0.0004
WESTSA     0.0003 0.0005 0.0000 0.0001 0.0003 0.0003 0.0007 0.0001 0.0000
COOR1      0.0000 0.0011 0.0001 0.0000 0.0000 0.0000 0.0013 0.0000 0.0002
COOR2      0.0000 0.0016 0.0003 0.0000 0.0000 0.0000 0.0018 0.0001 0.0004
COOR3      0.0000 0.0016 0.0003 0.0000 0.0000 0.0000 0.0018 0.0001 0.0004
HAWK1      0.0018 0.0000 0.0007 0.0013 0.0018 0.0018 0.0000 0.0012 0.0005
HAWK2      0.0001 0.0010 0.0001 0.0000 0.0001 0.0001 0.0012 0.0000 0.0002
HUNTER     0.0004 0.0004 0.0000 0.0002 0.0004 0.0004 0.0005 0.0002 0.0000
```

Table 4.4 Character state input file for MIX.

24 species, 29 characters

Name	Characters
Jamblycep	00000000010111101010000001010
Jvogleri-1	00000000001010101000000011010
Jvogleri-2	00000000001010101010000011010
Aoedogen-1	00011110001111010101001001110
Aoedogen-2	00101010001010101010000001010
Aoedogen-3	00000110000110101010001110010
Aoedogen-4	10000110000110010101001001110
Aoedogen-5	10000110000010010101001001110
Macleay-1	01100111010001000110110001001
Macleay-2	01101111110001000110110001001
Macleay-3	01101111010001000110110001001
Hawk-1	01101111010001000110110001011
Hawk-2	00000111010001000110110001011
Shark-1	01100011110001000110110001001
Shark-2	01100011010001000110110001001
Mand-1	01101111010001000110110001011
Mand-2	01100011010001000110110001011
West-1	01101111010001000110110001010
West-2	00100011010001000110110001001
Coor-1	01101011010001000110110001011
Coor-2	01101011110001000110110001011
Coor-3	01101111110001000110110001011
Carn-1	01100111110001000110110001011
Carn-2	01000111110001000110110001011

Appendix 5 Protocols followed for the isolation of mitochondrial DNA from *Argyrosomus hololepidotus*.

ALL MANIPULATIONS TO BE DONE ON ICE USING STERILE TECHNIQUES!

(a) Isolation of mtDNA following the rapid method published by Chapman and Powers (1984).

SOLUTIONS

TEK Buffer:

Tris (T-1378)	6.05g	(or	50mL 1M Tris-Cl pH7.5)
Na_2EDTA (ED2SS)	3.72g	(or	50mL 0.2M Na_2EDTA pH7.5)
KCl (Analar)	15g		
Milli-Q water	800mL		

Check pH. Make up to 1L

Autoclave in 100mL portions.

15% SUCROSE-TEK:

Sucrose (Analar)	75g	
TEK buffer to	500mL	

Autoclave in 100mL portions.

EQUILIBRATED PHENOL:

Phenol (Wako)	50mL (melt in 65^{0}C water bath)
0.1M Tris-Cl pH8	50mL (will require several 50 mL changes)
8-Hydroxy Quinolone (H-6878)	50mg

Stir gently whilst measuring pH. When pH stops climbing, separate phenol and mix with another 50mL buffer. After approximately 3 or 4 buffer changes, and when the pH is approaching .8 (7.8-7.9) store under the buffer protected from light at -20^{0}C.

NB. The yellow indicator dye will turn orange to red when phenol needs to be discarded. Once Wako phenol bottle is opened, store remainder under N_2 at -20^{0}C or even better at -70^{0}C.

Also require: 70% alcohol for cleaning, sterile water, 10% non-idet in TEK, 24:1 chloroform:isoamylalcohol, butanol, absolute alcohol.

EQUIPMENT

8 50mL homogenisers (Halu)

10mL, 5mL graduated pipettes and autopipettors

20 50mL high speed centifuge tubes

silanised pasteur pipettes, bulbs and silicon tubing

glass rods.

microcentifuge tubes and racks

Centrifuges- refrigerated benchtop: 50 mL swing out rotor (1000g, 4^0C) and microfuge rotor.

Sorvall or Beckman high speed: 50mL fixed angle rotor (18000g, 4^0C)

top loading balance

Gilson micropipettors- P1000, P200, P100,P20

hot water baths- 37^0C, 65^0C, and boiling.

PROTOCOL

(a) Isolation of mitochondria.

Weigh liver &/or gonad tissue samples on petri dishes (<10g).

	Tissue	Weight (g)
1.		
2.		
3.		
4.		

Finely mince the tissue using scalpels or scissors & transfer to glass teflon homogenisers. Homogenise in 5ml per gram of tissue in TEK buffer with one or two gentle passes with the pestle.

	Vol. of TEK(ml)
1.	
2.	
3.	
4.	

Filter homogenate through muselin into SS34 tubes. Carefully underlay homogenate with 10ml sucrose-TEK buffer. Balance the tubes with TEK buffer.

	Tube weight (g)	Balanced tubes.
1.		
2.		
3.		
4.		

Centrifugation:-Sorvall HB4 rotor (swinging bucket 50ml)

2100rpm (700g)/ 5 min/ 0^0C Time: On- Off-

Withdraw supernatant with a 10ml pipette using a valve bulb and transfer to clean SS34 tubes. Balance the tubes with TEK buffer.

	Tube weight (g)	Balanced tubes.
1.		
2.		
3.		
4.		

Centrifugation:-Sorvall SS34 rotor (fixed angle 50ml)

12800rpm (20000g)/ 20 min/ 0^0C Time: On- Off-

Discard supernatant, & if any gelatinous material is detected (that may be under the mitochondrial pellet) carefully remove using a spatula, **or** if pellet is not firm, resuspend in 20ml TEK buffer, balance tubes & repeat spin. Time: On- Off-

Discard supernatant and suspend pellet in 1ml TEK.

(b) Isolation of mtDNA.

Lyse mitochondria by the addition of non-idet to a final concentration 1% (lysis volume/9), 10 min at room tempurature in eppendorf tubes.

	Lysis vol (µl)	Non-idet (µl).
1.		
2.		
3.		
4.		

At room temperature extract twice with buffered phenol, once with chloroform, and then use butanol to reduce volume to <500µl.

Precipitate DNA using ethanol (2:1 100% ethanol), stand at -20^0C overnight.

	Ppt vol (µl)	Ethanol (µl).
1.		
2.		
3.		
4.		

Pellet DNA in eppendorf centrifuge: spin for 15 min at 4^0C. Draw off alcohol using a fine-tipped pasteur pipette, being careful not to dislodge the pellet. Cover tubes with parafilm and perforate, and dry pellet by vacuum dessication. Rehydrate at 100µl/g starting tissue for gonad, 20µl/g starting tissue for liver. Store at -20^0C until required for restriction digests.

(b) Isolation of mtDNA following a modification of Lansman *et al.*, 1981 (as detailed in Cahill, 1987).

SOLUTIONS

MSB-Ca^{++}

0.2M Na^{++}EDTA pH7.5

MSB-EDTA

1M sucrose with 5ml 1.5M sucrose

STE pH 8:

NaCl	0.58g
1M Tris-Cl pH8	5mL
0.2M Na_2EDTA pH8	2.5mL
Milli-Q water	to 100mL

Check pH and autoclave.

PROTOCOL

(a) Isolation of mitochondria.

Weigh tissue samples (liver/ heart/ gonad) on tared, glass petri dishes.

	Tissue	Weight (<10g)
1.		
2.		
3.		
4.		

Finely mince the tissue using scalpels or scissors & transfer to glass teflon homogenisers. Homogenise in 2ml of MSB-Ca^{++} per gram of tissue with one or two gentle strokes of the pestle.

	Vol. of MSB-Ca
1.	
2.	
3.	
4.	

Filter homogenate through muselin into SS34 tubes. Add 0.2M Na^{++}EDTA pH7.5 to a final concentration of 10mM. Balance the tubes with MSB-EDTA.

	Vol. of homogenate.(ml)	Vol. EDTA to 10mM.(ml)	Balanced tubes.
1.			
2.			
3.			
4.			

Centrifugation. Sorvall HB4 rotor (swinging bucket 50ml)

2100rpm (700g)/ 5 min/ 0^0C Time: On- Off-

Carefully decant supernatant into clean 50ml tubes,balance with MSB-EDTA & repeat spin. Time: On- Off-

Sucrose Step Gradient.

To prevent collapse of the soft cellulose nitrate tube the total volume should be 37.5ml (or 1.5mm from the top). Measure volume of supernatant from second low speed spin to calculate the volume of MSB-EDTA to be used.

	Vol. of supernatant(ml)	Vol. of MSB-EDTA(ml)	Balanced tubes.
1.			
2.			
3.			
4.			

Make the sucrose bilayer **immediately** before use!

Using a pasteur pipette joined by a piece of silcon tubing to 10ml graduated pipette, into the SW28 tubes gently underlay 10ml 1M sucrose with 5ml 1.5M sucrose. Overlay the supernatant using a long-nosed 5ml pipette, & balance tubes with MSB-EDTA.

Centrifugation. Beckman SW28 rotor (swinging bucket 40ml).

23000rpm (90000g)/ 45 min/ 0^0C Time: On- Off-

Being careful to take the mitochondria that have adhered to the walls of the tubes, extract the brown mitochondrial band at the interface of the two sucrose fractions using silanised pasteur pipettes (that have the tip bent to 90^0), & transfer to SS34 tubes.

Add approximately 3 volumes of MSB-EDTA, & balance the tubes.

Centrifugation. Sorvall SS34 rotor (fixed angle 50ml)

12800rpm (20000g)/ 20 min/ 0^0C Time: On- Off-

Discard supernatant, & if the mitochondrial pellet is not firm, resuspend in 20ml MSB-EDTA, balance tubes & repeat spin. Time: On- Off-

Suspend pellet in 3ml STE pH 8

(b). Isolation of mtDNA.

To lyse mitochondria and digest protein,

add:- 150µl 25% SDS (final concentration 1%)

60µl proteinase K (freshly prepared, 5mg/ml STE pH 8)

Incubate 37^0C/ 30 min.

Appendix 6 Details of restriction enzymes used, and the protocol followed, to digest mitochondrial DNA from *Argyrosomus hololepidotus*.

(a) Restriction enzymes

ENZYME NAME	RECOGNITION SEQUENCE
Ava-I	C/PyCGPuG
BamHI	G/GATCC
Bgl-I	GCCNNNN/NGGC
EcoRI	G/AATTC
HindIII	A/AGCTT
Pst-I	CTGCA/G
Pvu-I	CGAT/CG
Pvu-II	CAG/CTG
Xho-I	C/TCGAG

(b) Restriction digest protocol

NOTE: For 6 base cutters, use 20µl of mtDNA rehydration volume.
For 5.33 base cutters, use 30µl of mtDNA rehydration volume.

(i) Digestion mix

16.5µl	Sterile milliQ water (or appropriate volume to 50µl)
5µl	digestion buffer as appropriate to enzyme
5µl	BSA (bovine serum albumin)
20µl	mtDNA (or30µl for 5.33 base cutters)
2.5µl	RNAase (heat treated to inactivate DNAase activity)
1µl	restriction enzyme

Flick mix. Incubate at 37^0C, 2h. Flash spin.

(ii) Precipitation

30µl	3M sodium acetate pH 5.2
220µl	sterile milliQ water
600µl	absolute ethanol

Incubate on ice, 30min. Refigerated spin, 30min. Discard supernatant and dry under vacuum 30psi, 30min.

(iii) Mini-sub agarose gel electrophoresis

Rehydrate with 10µl 3 times loading buffer (3.LB). Vortex to mix, pulse spin. Load onto 1% agarose gel in TPE buffer, containing 1% ethidium bromide. Run at 60V, 1h. Destain in water.

Appendix 7 Protocol followed for sequencing sections of mitochondrial DNA from *Argyrosomus hololepidotus*.

(a) Total DNA extraction

DIGESTION SOLUTION:-

NaCl	100mM
Tris pH 7.5	10mM
EDTA pH 8.0	1mM

Therefore, in 100mls we have:

10ml 1M NaCl
1ml 1M Tris
2ml 0.5M EDTA

make up to 100ml with milliQ water

(i) Digestion

Add 0.5ml of digestion solution in an eppendorf tube.

Take a sample of the tissue of interest (liver, kidney, heart and muscle are best). The size of the sample need be no larger than half a fingernail. Place sample in a mortar and cover with liquid nitrogen. Grind the sample to dust using the pestle. Transfer the powder to the eppendorf tube containing digest solution.

Add:- 25µl of Proteinase K (10mg/ml). Shake well.

50µl of 10% SDS and mix gently.

Place in 55^{0}C water bath for at least 1 hour to digest (about 3hrs is best).

(ii) Extraction

Add 0.5ml of Phenol:Chloroform:Isoamylalcohol(25:24:1). Shake for about 5 minutes to ensure the phases are well mixed. Spin at 13000 rpm for 3 minutes. Pippette off the upper, aqueous phase and transfer it to a fresh tube.

Repeat this extraction process once using the P:C:I. mixture.

Perform the same extraction process twice using Chloroform:Isoamylalcohol (24:1).

(iii) Precipitation

Add 20µl 5M NaCl and 1ml 95% EtOH (cold). Place in freezer overnight.

Drain EtOH by decanting, remove trace amounts using a drawn out pasteur pipette and air dry tubes by leaving them open and inverted.

Resuspend pellet in a minimum of 50µl, and up to 300µl of milliQ water, depending upon the size of the pellet. Allow to resuspend in fridge (not freezer) overnight.

When DNA is fully resuspended store at -20^{0}C or -70^{0}C.

HINTS:- When transferring the aqueous phase to a fresh eppendorf make sure you don't get any of the lower organic phase. To achieve this without losing too much of the upper phase, first remove the lower phase with a pipette and discard. Spin the solution briefly. Using a wide bore pasteur pipette tip (you can buy these or make them by cutting back the ends of the normal tips) remove the aqueous phase. It is important not to take any of the interface. If there is still a whitish colour at the interface after two P.C.I. extractions, extract again until the interface looks relatively clear. (This process will draw any protein-bound DNA out of the organic layer.)

(b) Protocol for vertical acrylamide gel

(i) Solutions

ACRYLAMIDE SOLUTION: 40%

Acrylamide 38g
Bis Acrylamide 2g
in 100 mls milliQ water

These powders are toxic and should be weighed in the fume hood. The solution should be stored in the fridge and changed after about 2 months as acrylic acid tends to be formed.

TBE SHORT RUN BUFFER

Tris 108g
Boric acid 54g
EDTA 7.44g
in 2 litres of milliQ water

N.B. This buffer is used when sequencing near the primer.

TBE LONG RUN BUFFER

Tris 162g
Boric acid 27.5g
EDTA 7.44g
in 2 litres of milliQ water

N.B. This buffer is used when sequencing bases more than approximately 180 bp's from the primer.

The TBE buffer solution used depends on whether a short or a long sequencing run is performed.

GEL WORKING SOLUTION: 6%

Urea 36g
milliQ water 20mls
5C TBE buffer15mls
acrylamide solution 11.5mls

Make up the gel working solution in a 100ml beaker. Mix with a magnet at approximately 50^0C in a water bath. Filter the solution into a 250ml side arm flask.

De-aerate the solution for 5 minutes or until bubble formation significantly decreases. To this solution is added 450ml ammonium persulfate (4%: 40mg/ml) and 45ml TEMED. The solution should be poured as soon as possible after the Temed has been added.

(ii) Preparing glass plates for gel

THE THERMOSTATIC PLATE

Ensure plate is thoroughly clean and dry before commencing.

Wipe plate down with 95% EtOH in fume cupboard. Using a pasteur pipette put Repel Silane evenly over the plate (about two pipette loads is enough). Wipe over with tissue. Let dry (about 30 secs) then polish with tissues. Repeat this process three times. Wipe over 95% ethanol and polish. Store plate in such a way that it is protected from dust. Avoid touching the plate surface with anything.

THE UPPER GLASS PLATE

Prepare the following solution:

5mls absolute alcohol
130mls 10% acetic acid
13mls bind silane

Ensure glass plate is clean and dry.

Wipe plate over with 95% ethanol and polish. Pipette the above mixture evenly over the plate (make sure the notched side is up). Wipe over and polish. Wipe again with 95% ethanol. Wipe dry and polish. Store away from dust. Avoid contact with plate.

(iii) Pouring the gel

Protect working area with plastic sheets to catch any drips of toxic acrylamide solution. Using a spirit level and the thermostatic plate ensure that the gel pouring assembly is levelled.

Place thermostatic plate on gel assembly unit (raised, polished side up). Position spacers along the long edges of the gel with the cut out section of the spacers at the bottom and facing outwards (This is to accomodate the gaskets on the sequencing unit - have a look!). Place bulldog clips along spacers evenly. Ensure that clips do not cause buckling or warping of the spacers otherwise gel will leak out. Check that the clips are at least 21cm apart (across the plate). This distance must be maintained if the top, glass plate is to slide freely over the thermostatic plate when the gel is poured.

Place the upper, glass plate so that it bridges the thermostatic plate and the extended support platform of the gel assembly unit (the upper plate should extend to approximately the bottom of the spacer strips). The bind silane treated face of the upper plate should be facing down and the edge with the well in it facing toward the top of the thermostatic plate. Pour some gel solution near the top of the plate and allow it to seep between the two plates. Slide the top glass plate across the bottom thermostatic plate, adding more solution as necessary. Keep an eye on the bottom of the plates-if too little solution is present here air bubbles will form as the upper plate is moved forward.

When the leading edge of the upper plate (i.e. the edges on either side of the central well) are flush with the edge of the thermostatic plate, clamp the upper plate to the thermostatic plate using the bulldog clips. Position the shark tooth well former with teeth facing away from the gel. Make sure that there is excess solution at the top of the gel otherwise bubbles will form in the wells. The well former should not be pushed too far into the gel as this will make loading the gel almost impossible. About 5 - 7mm is fine. Position large clamp across the gel near the well former. The gel takes about 20 minutes to set (you can tell by the presence of haloes around any bubbles present in the gel) and should be used between two and twenty hours after setting.

If you plan to use the gel the day after it was poured then place moist tissues on either end and wrap in gladwrap to prevent it drying out.

HINTS:- Make sure that the gel apparatus, plates etc. are all set up before adding the ammonium persulfate and Temed to the gel solution. Once these products are added the gel must be poured immediately.

Try not to use plates whose surfaces are badly scratched as this can affect the quality of the gel.

If you find that you've got a huge bubble at the bottom of the gel after the two plates are flush, try pouring (or pipetting) any remaining solution along the bottom edge of the gel, before clamping the two plates together. If this is done slowly and carefully the size of the bubble may be reduced, and sometimes even disappear (with luck!).

Be careful as most of the chemicals used are toxic.

Always use gloves.

Use Temed in the fume hood.