FR&DC Final Report (FRDC 91/27)

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THE DISCRIMINATION OF YELLOWFIN TUNA SUB-POPULATIONS EXPLOITED WITHIN THE AFZ.

PHASE I: A PILOT STUDY TO DETERMINE THE EXTENT OF GENETIC AND OTOLITH MICROCHEMICAL VARIABILITY IN POPULATIONS FROM DIFFERENT PARTS OF THE PACIFIC AND INDIAN OCEANS.

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CONTENTS

1. **SUMMARY**

- Yellowfin tunas (n=111) from four western and central Pacific Ocean localities (Indonesia, Philippines, Coral Sea and Hawaii) were examined for otolith microchemical variability. Eighteen elements were examined, and significant geographic variation in the chemical signatures of the primordial region were found. Seven elements contributed most of this variation. Fish could be re-allocated to their catch areas with a correct classification rate of between 70 and 85%. Greatest similarities were observed between Hawaii and the Coral Sea, which may reflect the relatively cool spawning environments of these two areas.

- Yellowfin tunas from six Pacific Ocean localities (Philippines, Coral Sea, Kiribati, Hawaii, California and Mexico) were examined for five polymorphic allozyme loci and for variation in mitochondrial DNA (mtDNA). Significant spatial heterogeneity was detected for *GPI-1** (n= 426) but not for the other four allozyme polymorphisms (n=179-429) nor for mtDNA (n=434). *GPI-1*100* was the most common allele in western and central regions, *GPI-1*75* the most common in eastern samples. There was only weak evidence of genetic heterogeneity among the western/central sample.

- Both allozyme and mtDNA keys were developed for the routine genetic identification of white muscle samples of the six major commercial tunas from the Pacific Ocean.

2. BACKGROUND

The yellowfin tuna, *Thunnus albacares*, is a pan-tropical species and the subject of important fisheries in the Indian, Pacific and Atlantic Oceans. In 1992, an estimated 431 000 metric tons were taken from the western, central and southern Pacific region monitored by the South Pacific Commission (anon., 1993) while an estimated 259 000 tons were taken from the eastern Pacific (IATTC, 1992).

The yellowfin tuna fishery off Australia's east coast developed rapidly during the 1980s and in 1988-89 involved 45-170 boats catching nearly 1000 tonnes, worth in the order of $5 million (pers. comm. BRR 1990).

The Australian domestic yellowfin fishery was developed following the demise of the NSW southern bluefin fishery in the early 1980's and the "discovery" (up to 10 000 recreational fishermen had been enjoying the annual yellowfin runs for many years) that large and good quality yellowfin could be caught by trolling and small-scale longlining along the south coast of NSW. When Australian, "cold-water" yellowfin began to command hitherto-unheard-of prices on the lucrative Japanese fresh sashimi market, effort increased. In 1987, the AFS placed a moratorium on new endorsements in the ECTUNA fishery. In 1988-89, 45-170 boats were involved catching nearly 1000 tonnes. Today there are 292 endorsement holders, of which only 50-60 fish regularly (R. Miller AFMA pers. comm.). Throughout a broad cross-section of industry and management, there are now serious concerns about latent effort in the fishery.

Following the good catches of the 1980's, many operators, and in particular the longline fleet that take the bulk of the catch, invested heavily in the fishery. Bigger boats, more hooks, monofilament technology, satellite imagery and electronic aids all served to rapidly increase the effective effort in the fishery. At the same time, limits on the maximum size of boats permitted to fish in the historical fishing zone off southern NSW, an area in which catch rates have been high throughout the fishery, effectively restricted the domestic operations to inshore waters.

In offshore waters, a Japanese fleet, setting in the order of 7 million hooks per year (approximately 5 times more than the domestic fleet), takes 1500-3000 tonnes annually; 2-4 times the domestic catch.

The Australian domestic yellowfin tuna fishery extends from Cairns in north Queensland down the east coast to St Helens in Tasmania. With the exception of operations in Cairns, where yellowfin are taken throughout most of the year, the fishery is highly seasonal. In the 1990's it has concentrated on a "run" of small to medium-sized fish. These have been associated with southward incursion of the East Australian Current during spring and summer, and subsequent northwards retraction of the current during autumn and winter.

Anecdotal evidence (length records are not routinely kept in the fishery) suggests that since its beginnings in the mid-1980's, the average size of fish caught by domestic longliners has decreased markedly, to the point today where it would appear that the bulk of returns come

from recruitment of one or two year classes - probably the 1+ and 2+ cohorts. With this history, and recent claims of localised depletion of east coast stocks, the identification of the source of recruits to the fishery and the broader delineation of stock structure in the region has become a high priority to scientists and managers alike (Ward, 1990).

Whether or not the current domestic fishery exploits a separate, "Australian", sub-population, and how this may relate to the very large pool of recruits produced within the large Western Pacific "population", are considerations important in the formulation of any long-term development or management policy. For example, if yellowfin tuna exploited within the AFZ are part of a free-mixing Pacific stock then quotas applied solely to AFZ catches will have little conservation value to the Australian fishery unless similar restrictions are applied outside the AFZ. To date, decisions regarding management of the east coast yellowfin fishery have been made with little or no understanding of sub-population structure, interactions between domestic and foreign fisheries (both within and outside Australian waters) or the link between spawning grounds in the Coral Sea (within the AFZ) and recruits fished along the sub-tropical east coast of Australia. The shortcomings in our current knowledge of these issues prompted participants (including representatives from industry and scientific and management agencies) in the East Coast Tuna and Billfish Research Priorities Workshop held in Sydney in March 1990 to assign the question of "stock" delineation the highest priority for future research.

This need for improved information on possible stock structures led CSIRO to propose a pilot study investigating the feasibility of applying integrated data from two relatively new techniques (analysis of mitochondrial DNA and otolith microchemistry) and one established technique (allozyme electrophoresis) to the question of yellowfin sub-population structure. The need for a pilot study in this instance is clear-cut; before proceeding with a large-scale project, it is essential to assess both the suitability of the two new techniques for application to yellowfin tuna and the extent of genetic and otolith microchemical variability within the study area.

This pilot study also proposed the development of an allozyme-based taxonomic key that will allow for the first time the speedy and accurate identification of larvae, post-larvae and small juveniles of yellowfin tuna. This key should also permit the unambiguous identification of commercial tunas from which key morphological features, such as fins, have been removed.

The CSIRO proposal (incorporating both the pilot and follow-up projects) was detailed by John Gunn at the East Coast Tuna and Billfish Research and Monitoring Workshop held on 15-16 March 1990 and was also discussed at ECTUNAMAC.

The submitted proposal for the pilot study is included here as Appendix 3.

3. PROJECT DETAILS

3.1 OBJECTIVES

As the proposed project was designed as a pilot study to a subsequent larger research project dealing with the links between yellowfin spawning grounds in the Coral Sea and the recruitment to domestic and foreign commercial fisheries off the east coast of Australia, it had limited but well defined objectives. These were as follows:

1. To provide an allozyme-based taxonomic key to the early life history stages of Thunnus species occurring in eastern Australian waters. This key would provide, for the first time, an accurate and efficient means of separating larval, post-larval and small juvenile specimens of the principal commercial tuna species (e.g. yellowfin, bigeye, albacore, southern bluefin, skipjack).

2. To assess the genetic variability in larval/juvenile yellowfin tuna collected from four different locations; one off the north-east coast of Australia, two in other parts of the Western Pacific and one in the Indian Ocean.

3. Using the specimens for which genetic data have been collected, to assess the extent of variability in otolith microchemistry, particularly in that region of the otolith that is deposited during the early stage of larval development (i.e. while the larvae are close to the spawning area). Data on the variability among and within samples from the four areas will provide a means of assessing whether yellowfin tuna otoliths constitute a natural tag that will allow the identification of spawning areas from which recruits to domestic and foreign fisheries within the AFZ originate.

3.2 PROJECT PERSONNEL

Bob Ward	CSOF7	Co-Principal Investigator
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Tim Davis	CSOF7	Project Co-ordinator
Peter Grewe	CSOF5	Genetics
Nick Elliott	CSOF5	Genetics
Adam Smolenski	CSOF3	Genetics
Thor Carter	CSOF4	Otolith Chemistry
Naomi Clear	CSOF3	Otolith Chemistry
Song Chen	CSOF5	Statistics
Ian Harrowfield*		CSIRO Division of Mineral Products
Soey Sie*		CSIRO Division of Exploration Geosciences.

* Collaborating scientists at other CSIRO laboratories

4. RESULTS

4.1 VARIATION IN OTOLITH CHEMISTRY

Materials and Methods

Sampling

As the primary objective of this component of the project was to examine variation in otolith chemistry from yellowfin tuna spawned in different parts of the Western Pacific Ocean, small juveniles were collected from widely spaced locations: the Philippines, Indonesia, Hawaii and the Coral Sea (Fig. 1). Ideally, the experiment would have used otoliths of young larvae, which in all likelihood would not have moved more than a few miles from their spawning grounds. However, all historical collections of larvae contained specimens that had initially been fixed in formalin, a process that results in the dissolution of otoliths. With the exception of the Philippines, ichthyoplankton sampling throughout the region during the course of the project failed to collect adequate sample sizes.

Thus, on the assumption that small juveniles would not have moved significant distances from their spawning grounds, and certainly could not have moved between the sampling sites chosen, yellowfin smaller than 35 cm fork length, (i.e. of an age of approximately 120 days (Yamanaka, 1990)) were collected from two locations in Hawaii, one in the Philippines and one in Indonesia. In the Coral Sea, where yellowfin of this size were not available, samples ranged in size from 49-60 cm (300-400 days). In fish of this size, the potential for movement is much greater than those sampled from other parts of the Pacific and this was considered in the interpretation of the results. Table 1 provides details of the otolith and genetic sampling and specimens. Samples collected in 1992 were used only for genetic analysis, while only otoliths were taken from the Indonesian sample. Note that not all otoliths were analysed.

Otoliths were removed from fish either immediately after capture, or from frozen samples brought back to the laboratory. Once removed from the fish, otoliths were cleaned and stored dry in plastic SEM embedding capsules.

Preparation of otoliths

For both electron and proton probe analyses, careful and clean preparation of otoliths is essential. The methods used for sectioning, grinding and polishing otoliths are described in Gunn et al. (1992). At all stages of the preparation, otoliths were routinely cleaned in distilled water and, to ensure that no systematic contamination could occur, the order of preparation of otoliths was randomised.

To allow quantitative comparisons among specimens, all otoliths were sectioned and analysed along the post-rostral axis; a straight line from the primordium to the post-rostral apex. Either the right or left sagitta was chosen at random for preparation and analysis, and a subsample of otoliths pairs was analysed for examination of within-specimen chemical variation.
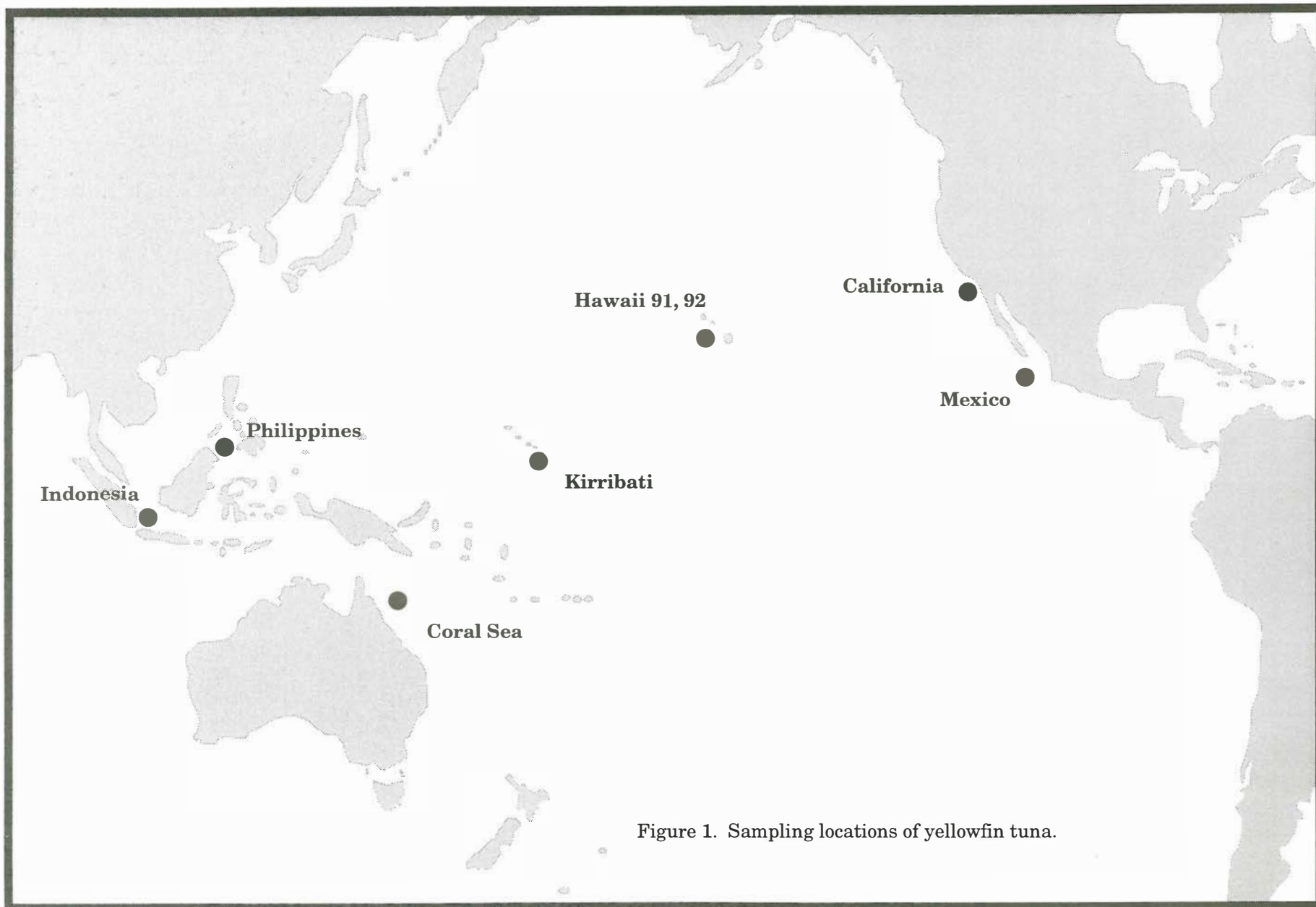
California

Hawaii 91, 92

Mexico

Philippines

Kirribati

Indonesia

Coral Sea

Figure 1. Sampling locations of yellowfin tuna.

Table 1. Sampling locations and sample details.

Location	Approximate lat./long.	Date	n	size[a]	genetics tissue[b]	otoliths taken
Indonesia	c.5°N 110°E	September 1991	20	32-35 cm	none	yes
Philippines	8°N 119°E	March 1991	47	32-46 cm	m,l	yes
Coral Sea	15°N 146°E	Oct./Nov. 1991	100	49-111 cm	m,l	yes
Kiribati	0°N 173°E	August 1991	29	52-67 cm	m	yes
Nikunau	2^{O}N 179°E	March 1992	35	not available	m	no
SW Baker Isle	2^{O}N 177°E	April 1992	9	not available	m	no
NW Nikumaroro	2^{O}N 176°E	April 1992	15	50-55 cm	m	no
Marshall Islands	5°N 173°E	August 1991	6	51-88 cm	m	yes
Hawaii-1	20°N 155°W	August 1991	17	120-180cm	o,t	no
Hawaii-2	20°N 155°W	October 1991	19	620-840 g	m,l	yes
Hawaii-3	20°N 155°W	November 1991	4	330-590 g	m,l	yes
Hawaii-4	20°N 155°W	December 1991	14	280-1020 g	m,l	yes
Hawaii-5	20°N 155°W	August 1992	80	c. 60-180 cm	m	no
Hawaii-6	20°N 155°W	August 1992	6	c. 60-180 cm	m	no
California	33°N 117°W	August 1992	41	c. 50-70 cm	m	no
Mexico	29°N 117°W	June 1992	40	not available	m	no

[a] lengths given in preference to weights, when available
[b] m=white muscle, l=liver, o=ovary, t=testis

Probe analyses - beam conditions

The chemical composition of otoliths was analysed using two probe microanalyzers - a Cameca "Camebax" wave dispersive electron microprobe located at the CSIRO Division of Mineral Products in Melbourne, Australia, and a proton probe located at the CSIRO Division of Exploration Geoscience in Sydney, Australia (see Ryan et al. for a detailed description of the latter instrument). The methods used in the wave dispersive electron microprobe analysis (WD-EMP) and proton induced x-ray emission spectroscopy (PIXE) of otolith matrices are described by Gunn et al. (1992) and Sie and Thresher (1992), respectively. Particular care was taken to avoid excessive damage to the otolith matrix in WD-EMP analyses. To ensure minimal damage, a beam energy less than 3.1 μWμm-1 sec-1 was used for all analyses.

Two separate experiments were conducted on the yellowfin otoliths.

(i) Experiment 1 - Spawning area discrimination - Geographic variation in the chemistry of early larval increments.

Both WD-EMP and PIXE analyses were used to characterise the chemistry of the early phases of the larval stage of each fish (i.e. when larvae are closest to the spawning ground). Otoliths from 150 specimens were prepared and analysed. The beam conditions used are summarised in Table 2. The order of analysis of otoliths was randomised to avoid systematic analytical error that can result from changes in spectrometer performance with time. Further safe-guards against such temporal effects included regular analysis of standards and verification of spectrometers.

Table 2. Beam conditions used in WD-EMP and PIXE analysis of yellowfin tuna otoliths.

Analytical Method	Analysis	Beam Diameter (μm)	Beam Current (nA)	Voltage
WD-EMP	larval increments	30	50	15 keV
	life history scans	14	20	15 keV
PIXE	larval increments	50	12.5	3 MeV

WD-EMP analyses on each otolith were made at two points, centred approximately 25 μm and 50 μm from the primordium along the post-rostral axis, using a 30 μm diameter beam. A single, 50 μm diameter PIXE analysis was made at a point centred approximately 37 μm from the primordium along the post-rostral axis (ie. centred approximately half way between the centres of points previously analysed with the electron probe). The analyses effectively examined the variation in otolith chemistry from days 5 and 12 in each fish's life.

In the very few cases (n=6) where small cracks prevented analysis on the predetermined positions, the beam was centred as close as possible to the positions without analysing the crack. The re-positioning overcame the possibility of analysing contaminants that sometimes accumulate in cracks (Sie and Thresher 1992), and also avoided the biases introduced by topographic irregularities into WD-EMP analysis of light elements such as sodium (Gunn et al. 1992).

(ii) Experiment 2 - Temporal/ontogenetic variation in the chemical composition of otoliths.

To examine ontogenetic variation in the concentrations of the major elements within the otolith matrix, WD-EMP life history scans (Gunn et al. 1992) were conducted on a subset of otoliths. For these scans, the beam was reduced to a diameter of 14 μm and transects conducted from the primordium to outside edge with 30 μm spacing between analytical points. The probe operating conditions were as detailed in Table 2.

Statistical analyses

Univariate statistical analyses were conducted using the commercial software packages Statview 4 and SYSTAT. The primary tools used in the multi-variate analysis of intra- and inter-site variation and in the examination of the utility of these data in the classification of yellowfin spawning sites were discriminant function (DFA) and canonical variate (CVA) analyses.

As differing approaches have been taken to the use of variables and the interpretation of results in recent studies in which DFA and CVA have been applied in the delineation of stock structure of fishes (e.g. Schaefer, 1991, Edmonds et al. 1991, CSIRO FRDC Final Report Project 1991/32), the current study also examined the statistical properties of these analytical techniques when applied to data on otolith chemistry. The objective of these examinations was to develop a rigorous approach on which future research could be based.

Results

Composition of yellowfin tuna otoliths

In analyses of 150 yellowfin otoliths, 19 elements (Ca, C, O, Na, Sr, K, S, Cl, Fe, Ni, Rb, Zn, Mn, Cu, Br, Sn, Hg, Pb, Th) were detected at or above the minimum detection limits of the two analytical methods. Fig 2 shows mean concentrations and standard deviations around these means for these elements across all samples. Calcium, sodium, strontium, potassium, sulphur and chlorine, all of which were present at concentrations in excess of 100 ppm, were analysed using WD-EMP. PIXE was used to measure strontium, calcium, and the trace elements - mercury, copper, iron, zinc, bromine, lead, rubidium, manganese, nickel, thorium and tin, which were present at concentrations of 0-20 ppm.

In relatively few of the otoliths examined were nickel, bromine and lead present in concentrations above the minimum detection limits. There is some doubt regarding the reliability of detection of such rare occurrences (Soey Sie, CSIRO Division of Geoscience Exploration, pers. comm.), and in some cases the incidental occurrence of elements such as nickel or molybdenum can indicate contamination during preparation (Sie and Thresher, 1992). For the purposes of multivariate analyses however, we chose to consider these elements. In fact, in DFA and CVA they provided little or no contribution to discrimination of sites and their if they were excluded from the data set it would have no significant effect on the analyses or the conclusions drawn.

The concentrations of the majority of elements were normally distributed across the "population" (ie. across all sites). The exceptions were thorium, in which the distribution was bimodal, and chlorine, in which there was significant skewing of concentrations to the right. We have been able to find no explanation for the bi modality in thorium concentrations, either from a biological or analytical standpoint and as bimodality was thought likely to be an artefact thorium values were excluded from analyses. The pattern in chlorine concentrations is similar to that seen in many other fish species analysed by CSIRO over the past five years (e.g. morwong - FRDC Final Report 91/32, Southern Bluefin Tuna - unpubl. CSIRO data). Comparisons of the chemistry of left and right otoliths from the same fish, and randomising

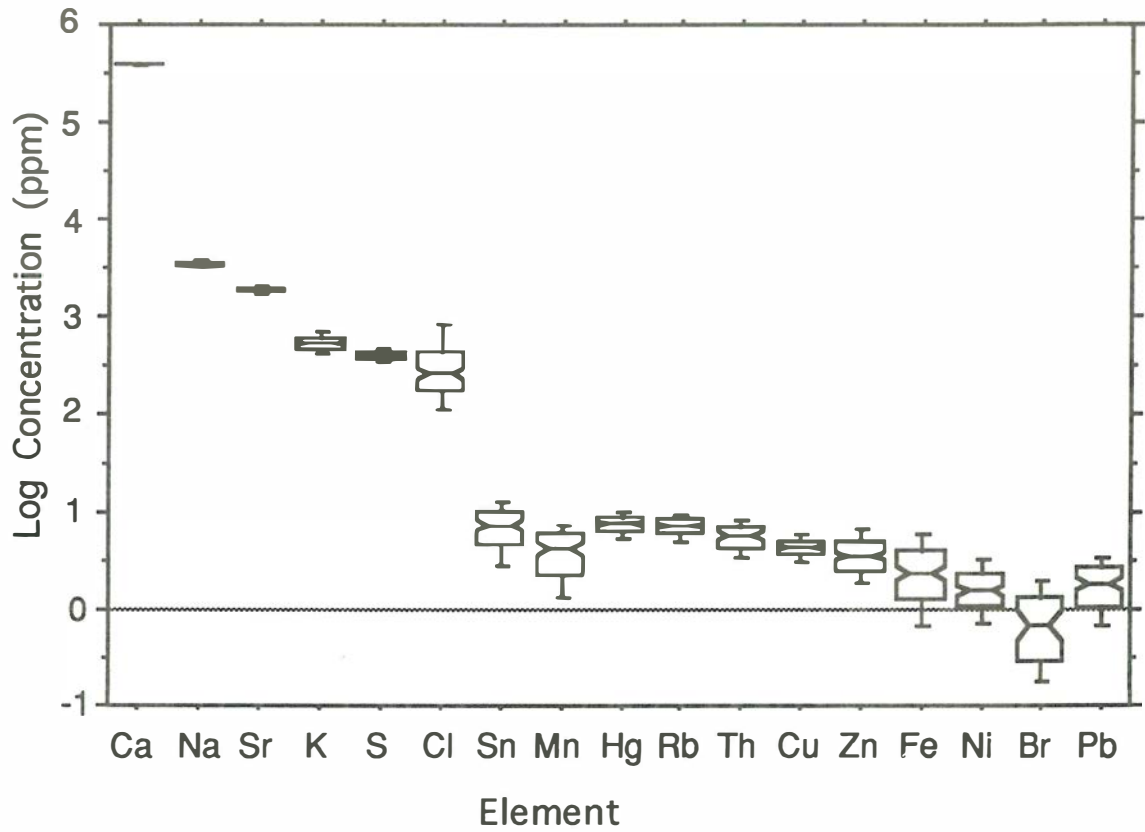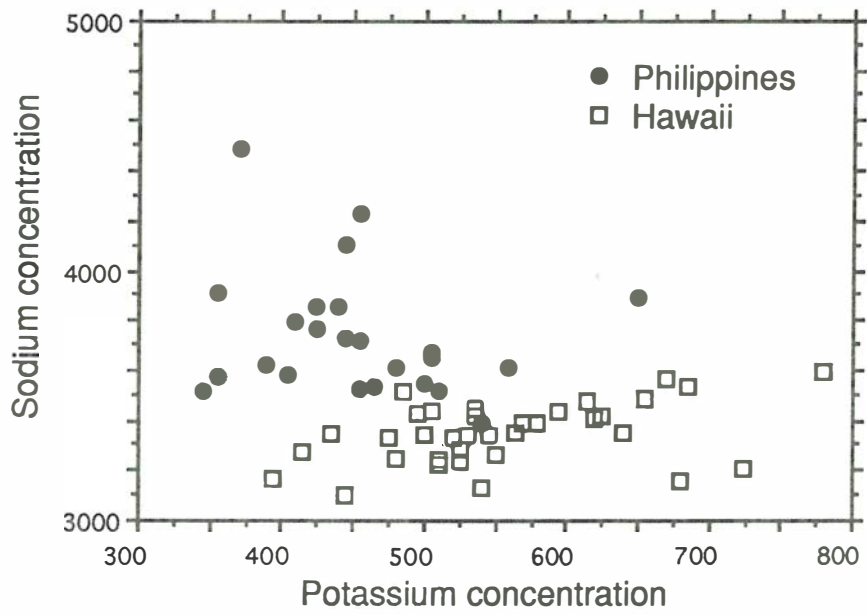Fig. 2. Mean, SD and range of concentrations of elements in yellowfin tuna otoliths.

Fig. 3. Sodium : potassium relationships for Hawaiian and Philippines samples

the order of preparation and analysis of otoliths have confirmed that the high chlorine levels in yellowfin are a associated with variable levels in the otolith matrix, rather than as the result of contamination.

Relationships between elements within yellowfin tuna otoliths

There are significant relationships in the concentrations of the six most abundant elements in the otolith matrix (Table 3). Of particular note are the relationships between sodium and chlorine, calcium and sulphur, and calcium and chlorine levels. The relative concentrations of these elements show clear geographical linkages; e.g. in a comparison between otoliths from the Philippines and Hawaii, a plot of sodium versus potassium concentrations reveals almost total separation of samples (Fig 3).

Table 3 Relationships between major elements within the otolith matrix of yellowfin tuna otoliths

	Calcium	Strontium	Potassium	Sulphur	Chlorine
Strontium	NS				
Potassium	NS	NS			
Sulphur	0.002	0.046	NS		
Chlorine	0.001	NS	0.017	NS	
Sodium	NS	0.040	NS	NS	<0.0001

Geographic variation in chemical composition

As sample sizes from the Marshall Islands and Kirribati collections were small, univariate and multivariate statistical analyses of geographic variation in chemical composition were restricted to otoliths collected at the four major sampling sites; the Coral Sea, Philippines, Hawaii and Indonesia.

(i) Univariate analyses

Univariate analysis (ANOVA with SNK post hoc testing) showed significant inter-site variation in the concentration in 6 of the 18 variables considered (Table 4 and Fig 4), with strontium exhibiting significant variation in both the PIXE and WD-EMP data.

Most notable among the varying elements were significantly higher concentrations of sodium and chlorine, and lower concentrations of potassium in the Philippines than in any of the other sites; higher zinc in the Indonesian samples than in those from other sites; generally lower levels of strontium in the Coral Sea and Hawaii; and high mercury levels in the Philippines and the Coral Sea.

Figure 4. Geographic variation in the concentration of elements in yellowfin tuna otoliths
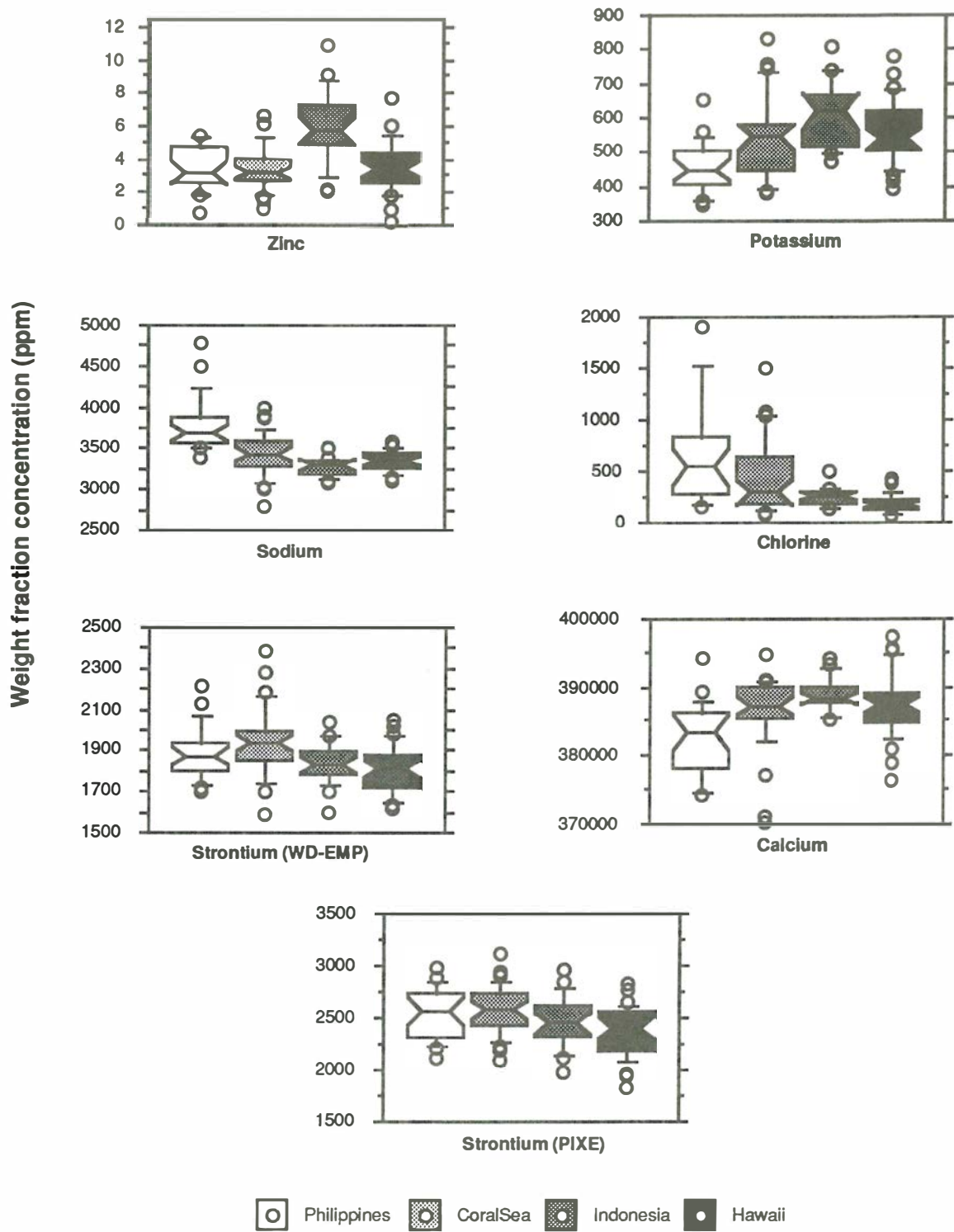
Table 4. Elements within the otolith matrix of yellowfin tuna showing significant geographic variation.

Analytical Method	Element	F-value	Significance Value
PIXE	strontium	4.788	0.0036
PIXE	zinc	4.251	0.0070
WD-EMP	calcium	8.257	< 0.0001
WD-EMP	chlorine	7.946	< 0.0001
WD-EMP	potassium	10.574	< 0.0001
WD-EMP	sodium	24.231	<0.0001
WD-EMP	strontium	6.199	0.0006
WD-EMP	sulphur	2.668	not significant (0.0514)

(ii) Multivariate analyses - Discrimination analyses

A detailed report on this component of the study is provided in Appendix 1. The summary below provides a brief outline of the key elements of the analyses.

The data set used for discrimination analyses consisted of chemical data from four sites - Hawaii, Coral Sea, Philippines and Indonesia - providing a total of 111 specimens. Initially the full data set of 18 variables (elements) was considered.

The high dimensionality of the data set renders graphical representation of the differences among sites difficult. Table 5 provides pairwise Mahalanobis distances among the four sites - a measure of relative similarity of the groups. From these it is evident that on a relative basis the Philippines is an "outlier", clearly separated from the other three sites. At the other end of the scale, Hawaii and the Coral Sea, and Hawaii and Indonesia are relatively close to each other. Although the estimation of Mahalanobis distance is only a first step, these patterns of similarity are repeated to at least some extent throughout the multivariate analyses.

Table 5. Pairwise Mahalanobis distances among groups, based on the complete data set of 111 individuals and 18 variables.

Hawaii	0			
Philippines	15.26	0		
Coral Sea	4.09	8.78	0	
Indonesia	4.79	25.05	10.225	0
	Hawaii	Philippines	Coral Sea	Indonesia

The high level of dimensionality in the data set - ie the relatively high number of variables compared with the sample sizes for each site - has the potential to cause major problems in discriminant analyses. The performance of discrimination techniques such as linear discriminant function analysis, is measured by the relative misclassification rates. Misclassification rates typically fail to improve once the number of variables reaches a threshold, a feature termed the "peaking phenomenon". To avoid this problem, the

dimensionality of data sets can be reduced either by restricting the number of variables considered or by projecting data into a lower dimension space. To examine the relative merits of these two approaches, we used both methods in considering the yellowfin data.

Selection of key variables was conducted using canonical variate analysis (CVA) (Rao, 1973) and normal distribution-based stepwise variable selection procedures. The results of the CVA (Table 6) suggest seven variables ; Zn and Ca measured by PIXE, and Na, K, Sr, Cl, and Ca measured on the WD-EMP, play the most important roles in separating the four groups.

Table 6. Total-sample standardised canonical coefficients. Variables considered most important in the separation of sites are marked with an asterisk.

Element	$\gamma1$	$\gamma2$	$\gamma3$
Sodium *	1.606	0.357	-0.020
Strontium (WD-EMP) *	0.601	-0.140	0.491
Potassium *	-0.778	-0.064	0.085
Sulphur	-0.023	0.117	-0.289
Chlorine *	-0.650	0.046	0.139
Calcium (WD-EMP) *	-0.558	-0.122	0.109
Iron	-0.040	0.324	0.300
Nickel	0.168	-0.152	-0.276
Rubidium	-0.004	-0.389	-0.090
Zinc *	-0.317	0.806	0.622
Calcium (PIXE) *	-0.046	0.466	-0.236
Manganese	0.016	0.446	0.066
Copper	-0.027	0.028	-0.068
Bromine	0.020	-0.167	0.290
Strontium (PIXE)	0.201	-0.076	0.233
Tin	-0.098	0.225	0.010
Mercury	0.232	-0.047	0.429
Lead	-0.087	-0.175	-0.297

The normal distribution-based stepwise procedure uses forward selection and backward elimination of variables. It assumes the population groups are multivariate normal distributed with a common covariance matrix. Fig. 5 gives QQ plots of Mahalanobis distance for each group. The basically linear shape of these plots for each of the four sites provides confirmation of the assumption of normality in the data. The results of the stepwise procedure (Table 7) are very similar to those of the CVA, with one additional variable, strontium measured with PIXE, being added to the list of important discriminators.

Significantly, in both variable selection procedures, sodium, zinc, potassium and strontium rated as the four most important discriminators.

Fig. 5. QQ plots of the Mahalanobis distances between the four site groupings.
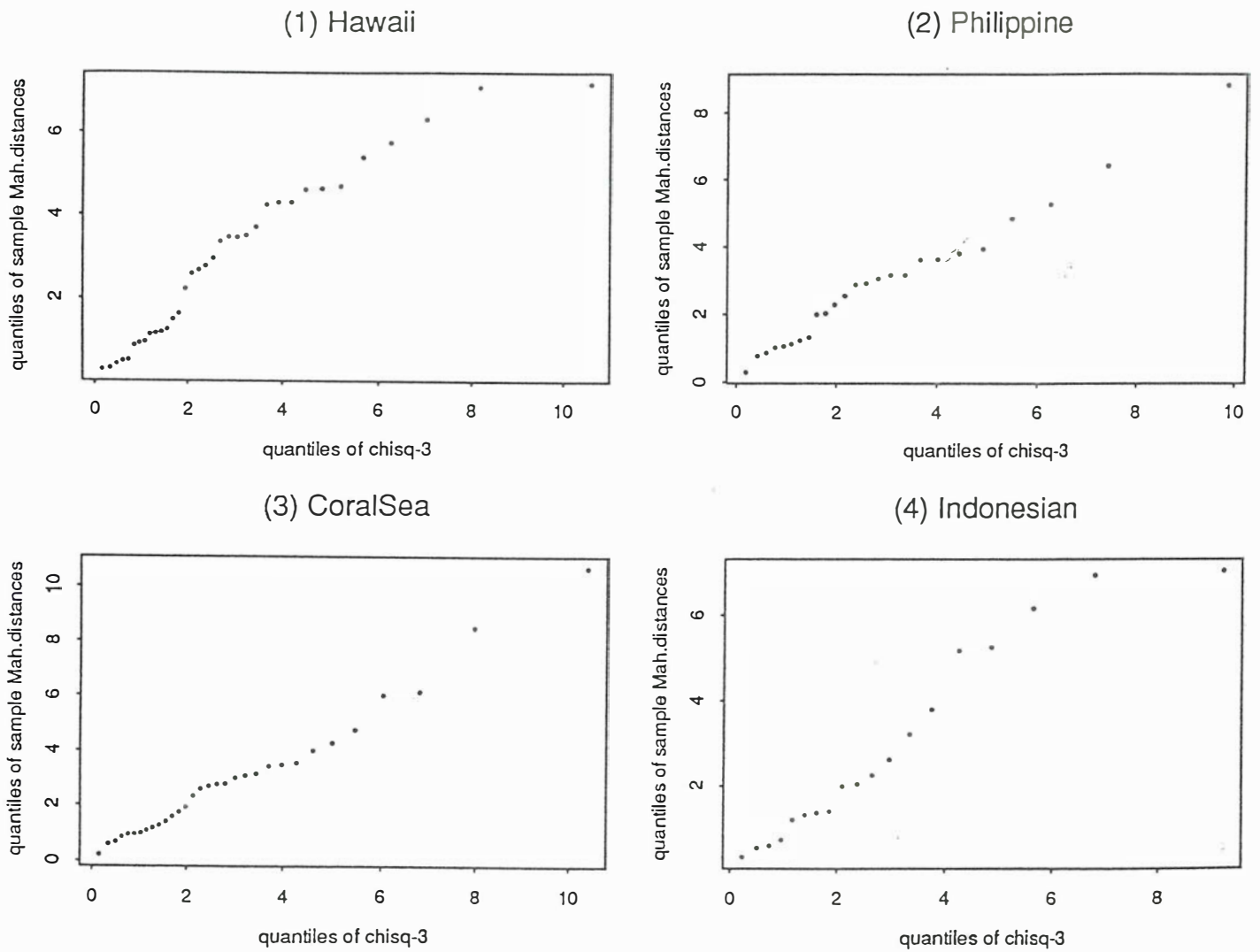
Table 7. Variables selected by the stepwise procedure.

Step #	Variable entered	Variable removed	F-statistics	P>F	Wilk's lambda	P<lambda
1	Sodium	nil	26.022	0.0001	0.578	0.0001
2	Zinc	nil	12.822	0.0001	0.424	0.0001
3	Potassium	nil	12.278	0.0001	0.314	0.0001
4	Strontium (WD-EMP)	nil	10.987	0.0001	0.238	0.0001
5	Calcium (WD-EMP)	nil	5.311	0.0019	0.207	0.0001
6	Chlorine	nil	4.424	0.0058	0.183	0.0001
7	Calcium (PIXE)	nil	3.261	0.0246	0.167	0.0001
8	Strontium (PIXE)	nil	2.846	0.0414	0.154	0.0001

Using the reduced data set (i.e. the 8 variables selected above), an assessment was made of the influence of the "peaking phenomenon" on total misclassification rates in a linear discrimination analysis. A detailed description of the results of this analysis are provided in the appended report. However, in brief, there was significant evidence of the peaking phenomena in the data set, with the most suitable number of variables being the eight indicated in Table 7. Using these variables, the total misclassification rate was 25.9%.

The final stage in the discriminant analysis of the otolith data was to examine whether it was possible to reduce further the total misclassification rate through data projection. The projection of the eighteen variables into a three dimensional data set was achieved by transforming data points for each of the 18 variables (elements) into the three dimensions of the raw canonical coefficients. The projected data are shown by way of three scatter plots in Fig. 6. These plots show clearly the separation of the Philippines in (1) and (2), the Coral Sea in (3), and Hawaii and Indonesia in (2). The projected data show clear separation of sites (Fig. 7) and produce a total misclassification rate of 19%, much lower than the 26% achieved using the unprojected data.

The classification performance of the projected data, examined on a site-by-site basis (Table 8), shows very low levels of misclassification for all sites except Hawaii (29.6%). Significantly, the majority of misclassifications of Hawaiian samples are towards the Coral Sea, and of five Coral Sea fish misclassified, four were grouped as Hawaiian.

Table 8. DFA classification matrix based on three dimensional projected data (% in parentheses).

Hawaii	25 (71.4)	0 (0)	6 (17)	4 (11.4)	35
Philippines	1 (4)	21 (84)	3 (8)	0 (0)	25
Coral Sea	4 (12.5)	1 (3.1)	27 (84.4)	0 (0)	32
Indonesia	3 (15.8)	0 (0)	0 (0)	16 (84.2)	19
	Hawaii	Philippines	Coral Sea	Indonesia	TOTAL

Fig. 6. Scatter plots of projected data with group membership as follows : p = Philippines, h = Hawaii, i = Indonesia, and c = Coral Sea.
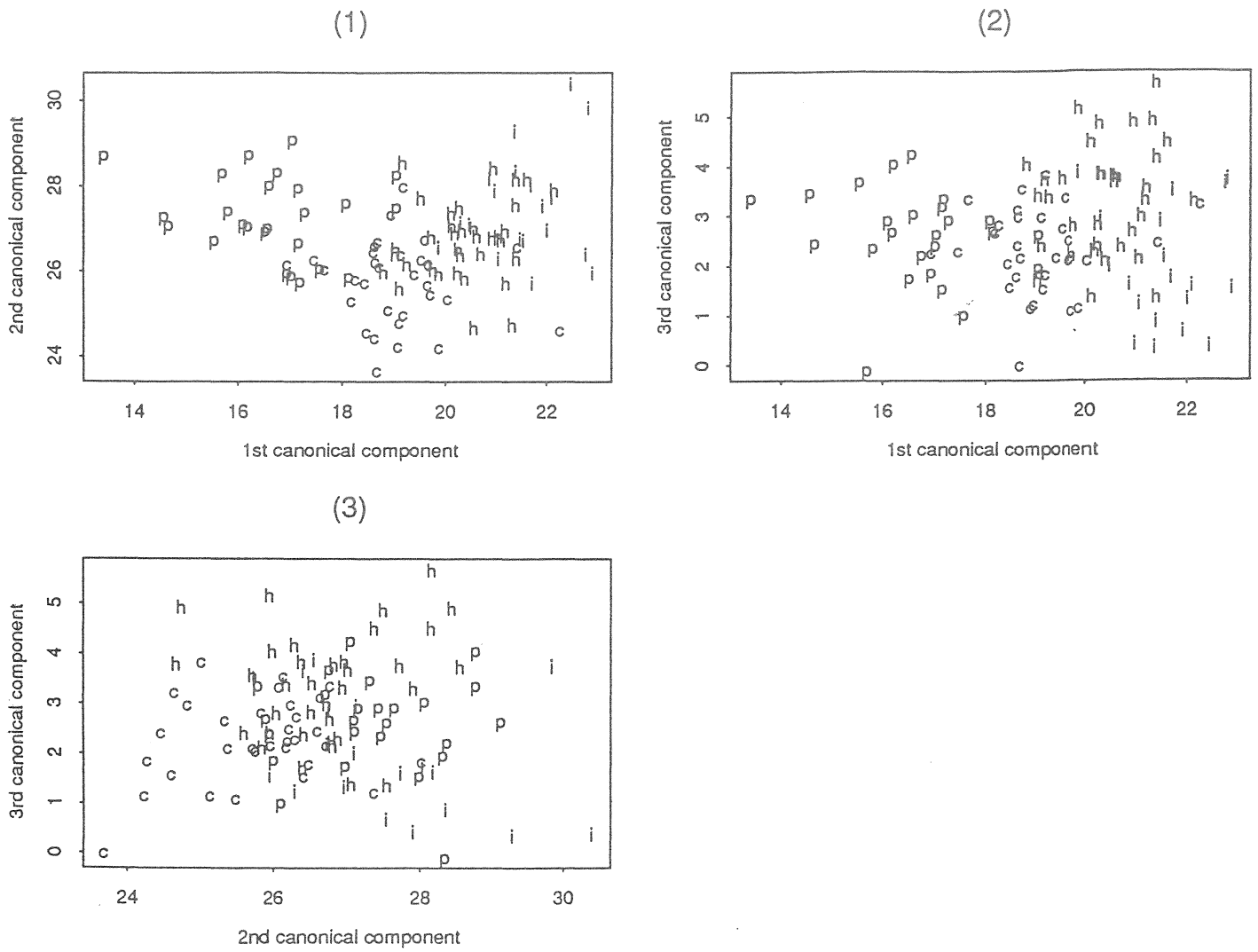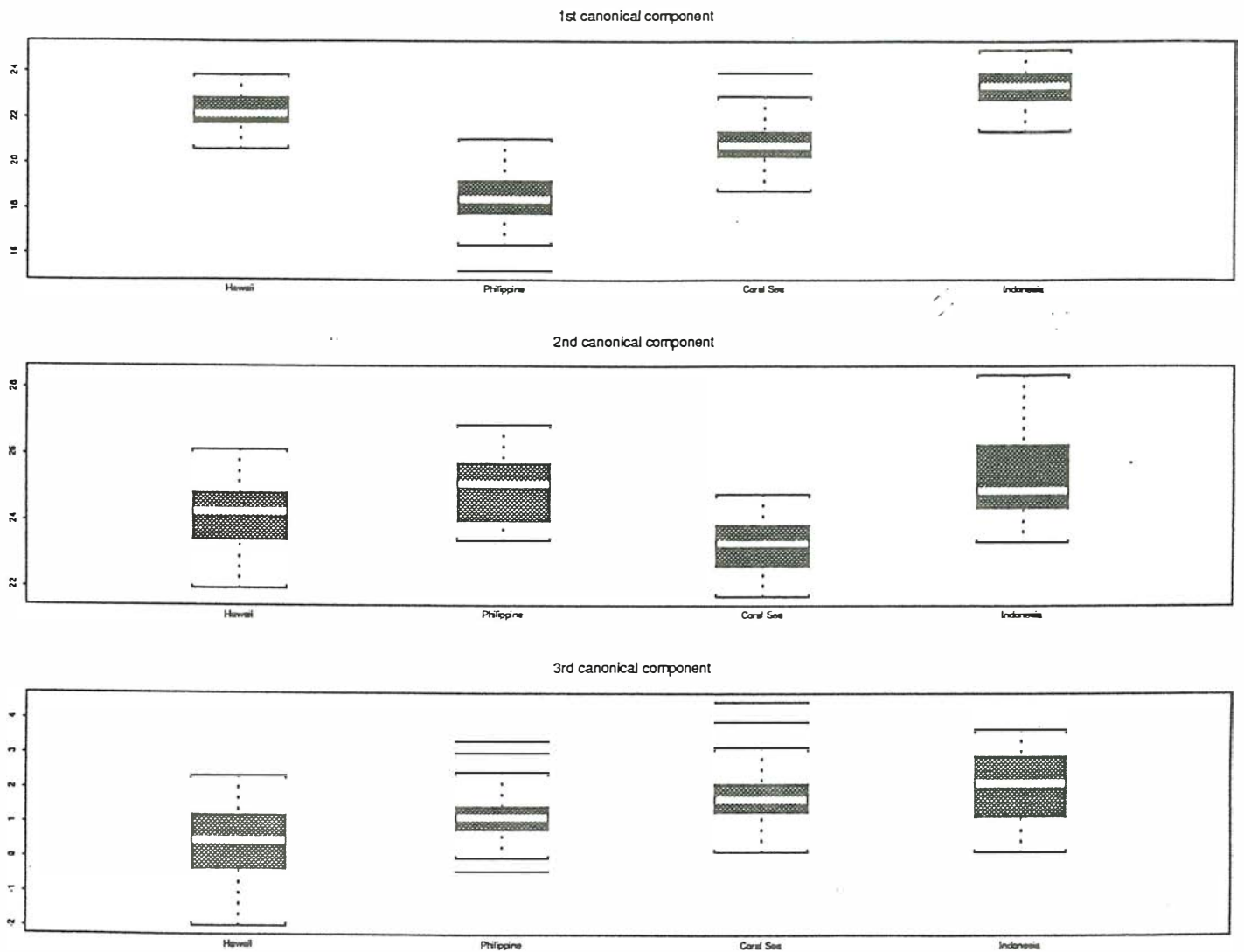
(1)

(2)

(3)

Fig. 7. Box plots of projected data showing significant differences among sites.

Ontogenetic variation in the composition of otoliths

As these analyses were by-and-large outside the primary objectives of the project, and microprobe time was limited, only small numbers of life history scans were conducted. The principal objectives of this work were, first, to examine how concentrations of elements changed during the early life history of yellowfin and second, whether fish collected from the same site exhibited similar patterns of variation.

Fig. 8 provides an illustration of the extent of ontogenetic variability in two specimens from the Philippines and two from Hawaii, all approximately 100 days old.

Discussion

Yellowfin tuna otoliths are similar in chemical composition to the otoliths of many other fish species examined over the past 6 years by CSIRO and by other laboratories using both microprobes and bulk analytical techniques (Gunn et al 1992, Sie and Thresher 1992, Edmonds et al 1991, Campana and Gagne 1992). In addition to calcium carbonate (calcium, carbon and oxygen), the primary constituents of the otolith matrix, in descending order of importance, are sodium, strontium, potassium, sulphur and chlorine. All of these elements are generally present in concentrations of greater than 100 ppm and can be measured using WD-EMP. Using PIXE techniques it is possible to measure accurately the concentrations of a suite of microconstituents, present at concentrations from 1-10 ppm. It is likely that other elements are also present at concentrations below 1 ppm (e.g. Campana and Gagne 1992). However, it is not practical to measure these using either WD-EMP or PIXE.

Using either WD-EMP or PIXE, combined with careful sectioning and preparation of otoliths, it is possible to examine and compare among specimens the concentrations of these elements at predetermined life history stages. In this study we examined the variation in concentrations of elements in the region close to the primordium. This region of the otolith is laid down shortly after a larva hatches from the egg, during the first few weeks of larval life. For the purposes of this study, it has been assumed that in the first couple of weeks of life there has been no large-scale advection of the larvae and thus that the region we have analysed was laid down while the larvae were within the general area in which they were spawned. Our objective was to examine the extent of variation within and among sites, and in so doing assess whether a site-specific spawning signal was evident in the otolith chemistry.

The key hypothesis within this pilot study, and other similar studies in which otolith chemistry has been used as an indicator of sub-population or stock affinity (e.g. Thresher et al, in press, Campana and Gagne, 1992, Edmonds et al 1991), is that otolith chemistry varies in response to spatial and/or temporal variations in physical and/or biological conditions within a fish's environment. There is a growing body of literature that supports the conditional acceptance of this hypothesis (Kalish, 1989, 1990, 1991, 1993; Radtke, 1989; Radtke et al., 1990; Radtke and Shafer, 1992; Secor et al., 1993). However, the clear message from most of these studies, is that variation in the concentration of many elements is not linked in a simple or
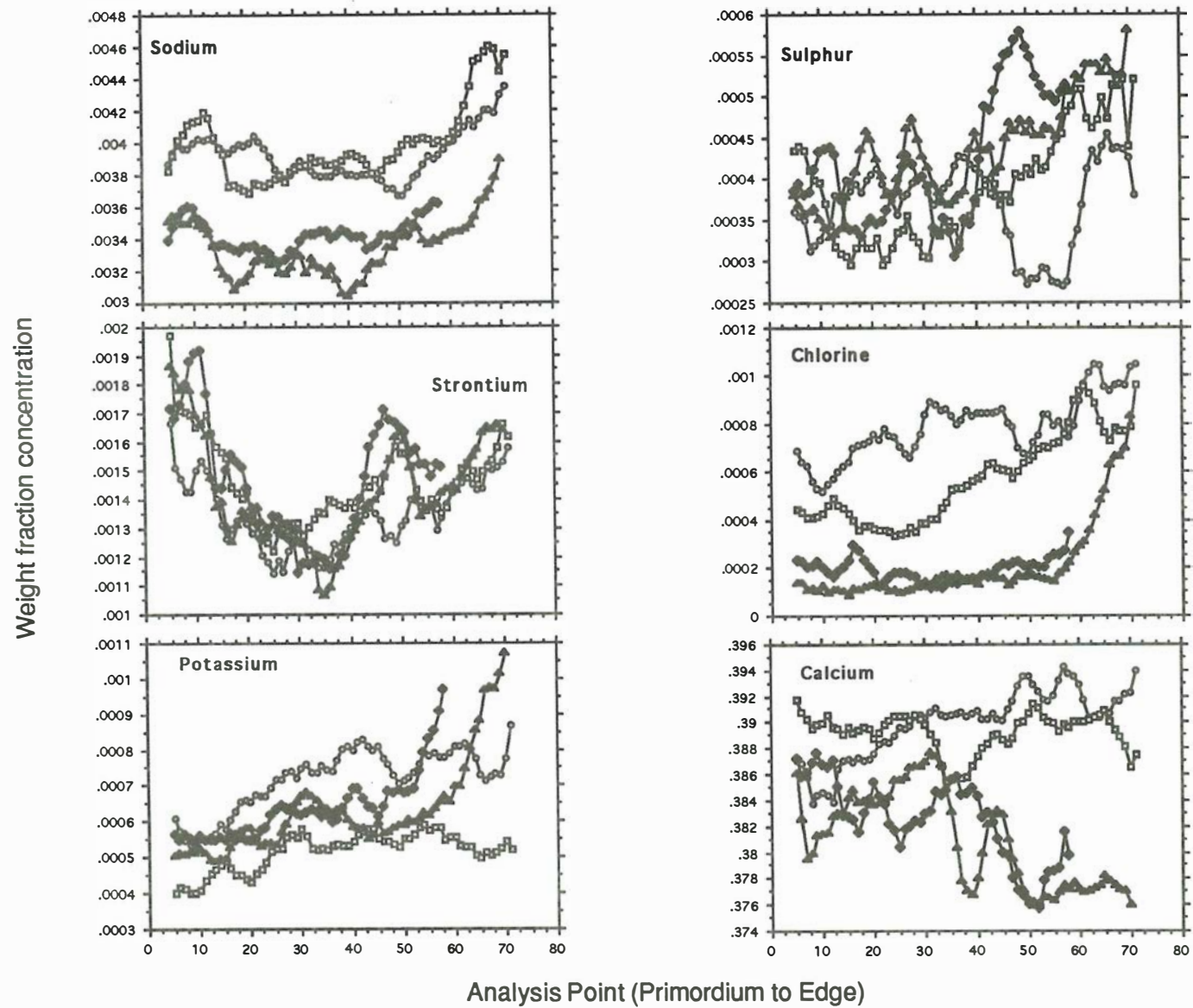
Fig. 8. Life history scans of otoliths from two specimens from the Philippines (□ and ○) and two from Hawaii (● and ▲).

direct way to variations in one environmental factor (e.g. temperature). The more we learn of what controls or influences otolith composition, the clearer it becomes that environmental effects are likely to exhibit both direct and indirect effects on the physiology of fish and ultimately on the chemical composition of otoliths.

While this is likely to complicate any attempts to use the concentrations of elements within the otolith matrix in back-calculation of environmental conditions (see e.g. Radtke et al., 1990), in the context of stock delineation or identification of spawning grounds, the primary interest is in whether the net effect of physical and biological environmental conditions, physiological responses, and perhaps genetic effects, produce an area- or site-specific signal.

Over broad geographic ranges such as those relevant for Pacific yellowfin tuna, it seems reasonable to expect that, for any given period, variations in physical and biological conditions among spawning areas will be greater than within individual spawning areas. Thus, if the key hypothesis regarding environmental influences on otolith chemical variation is valid, otolith chemistry should vary more among sites than within a single site.

The data from this pilot project suggest that this is the case in Western Pacific yellowfin tuna. Significant geographic variation in the chemical composition of larval portions of otoliths provides a positive indication that these data may be useful in determination of the spawning origin of fish. Low misclassification rates in CVA- and DFA-based discrimination of samples from different sites provide further encouragement. However, it is important to stress that the pilot project set out to compare widely-spaced spawning locations. It was thought that if this order of comparison revealed non-significant levels of variation, there would be little point in continuing. The significant variation is a positive outcome, one that builds on the microprobe-based stock delineation research of Thresher et al (in press) on jackass morwong and SBT (Thresher et al., in prep.) and of that of Calaprice (1985) on Atlantic Bluefin Tuna and yellowfin tuna in the eastern Pacific (Calaprice, unpubl. data), At the same time however, some significant questions remain to be answered before the variation seen in yellowfin tuna can be employed in quantitative attempts to delineate spawning origin of individual fish and the stock structure of populations.

First, the level of fine-scale geographic variation in yellowfin tuna chemistry needs to be examined. Campana and Gagne (1993) have reported significant variation in the chemistry of otoliths from cod collected over scales of tens to a few hundred miles. However, their study was based on bulk analytical techniques and no attempt was made to examine individual year classes. Consequently, the otolith material they analysed will have been laid down over long and possibly variable time periods. It is also possible that differences among sites may have been affected by differences in age composition of samples. Regardless of these complications, it is clear that a number of demersal fish (e.g. orange roughy (Edmonds et al., 1991) and morwong (Thresher et al., in press)) show variation in otolith chemistry over small geographic scales. The reasons for this are not clear. However, it may be that the variation in cod and other demersal fishes is related to their essentially sedentary life styles in environments where significant heterogeneity in topography, physical oceanography and biological systems could

all drive differences in fish physiology. In migratory and pelagic fishes such as tunas such factors are likely to be less significant and thus we may expect less fine scale variation.

In the current study, even the very small yellowfin sampled in Hawaii, Indonesia and the Philippines had the potential to move significant distances from their spawning grounds. In the case of the Coral Sea samples, comprising fish in the order of 300 days old, the potential for movement was even greater. It is perhaps surprising then that geographic, or site, affinity among these samples was significant and allowed clear discrimination of the sites. Such affinity suggests that fine-scale variation in tunas may be less marked than in ground fishes. Nevertheless, until structured sampling and analysis is conducted, the scales of geographic variability will not be resolved, and the potential for use of the techniques and data in identification of spawning grounds and stock will be open to question.

A second major unknown in the application of otolith data to stock identification is the temporal stability of the chemical signals being used to discriminate among fish, spawning grounds or stocks. Samples taken from different locations at one time may differ significantly, but would similar differences be seen a month later or in another season? Similarly, does otolith chemistry in fish from one site, or area, vary significantly between years? If so, it may be necessary in examining geographic variation to restrict comparisons to single cohorts. The limited information on temporal variation in yellowfin otoliths provided by life history scans suggests significant variability is common in all of the major constituents. However, it is not clear whether these observations of variation in individuals can be extrapolated to the population level, particularly as the effect of ontogenetic changes in physiology may play a significant role in such variation (Kalish, 1989).

Much of what remains unknown regarding fine scale variation and temporal stability in otolith chemistry has been ignored by previous studies of otolith chemistry or has been beyond the scope of the many short term projects. However, it is clear that if these questions are to be resolved and a valid assessment of the utility of the techniques made, sample sizes will need to be large enough to allow rigorous statistical analysis and testing of hypotheses, and sampling will have to be carefully structured to allow resolution of the issues.

A spin-off to the principal results of this study has been the chance to integrate data from two microprobes and to evaluate the most suitable technology for analysis of otolith chemistry. CSIRO began its experimentation with microprobe analysis of otoliths in 1987 using a WD-EMP (Gunn et al. 1992). It has been shown that this instrument measures accurately the concentrations of the six most abundant elements (except carbon and oxygen) in the otolith matrix, and it has since been used widely to examine environmental and physiological influences on the chemical composition of otoliths. As the focus of otolith studies moved towards stock discrimination, proton probes were suggested as a means of providing data for many of the microconstituents within the otolith matrix, which were present in concentrations of 1-20 ppm. The general philosophy was that the larger the suite of elements examined, the more chance there would be of finding elements that varied among areas and stocks. In yellowfin otoliths, while the suite of elements was comparable to those found in the otoliths of other species, only zinc, of all the microconstituents, varied to the extent that it played a

significant role in discrimination. Statistical analysis also revealed the effects of the "peaking phenomenon" when too many variables are thrown into the discrimination procedure. At least for the case of yellowfin, but potentially also for other species, the results suggest caution in the "more is best" approach and perhaps that the cost effectiveness of PIXE or similar high-sensitivity analyses should be evaluated before they are employed on a regular basis.

In the course of the statistical analyses of the yellowfin data, it was clear that the greatest level of similarity among the samples was between Hawaii and the Coral Sea. As it can be safely assumed that the fish from the two areas did not have the opportunity to mix, the reasons for this similarity are not obvious. It seemed unlikely on the basis of what is known about the forage resources of the two areas that the fish were eating exactly the same food. Similarly the salinity levels of the two areas differ significantly. However, the sites do fall on the northern and southern extremities, respectively, of the spawning range of the species. A reasonable hypothesis for the similarity may then be that fish from these higher tropical latitude spawning grounds exhibit a peculiar otolith chemistry, perhaps as a result of the lower water temperatures at which spawning and larval development takes place. As the influence of water temperature of the elements analysed by WD-EMP in particular is likely to be indirect, we think it may be useful to examine whether the latitudinal differences show up in other constituents that may be more directly linked. O18:O16 ratios, in carbonates, are known to be closely related to temperature in which the carbonate matrix is deposited. If geographic differences in O18/16 ratios within the otolith matrix can be detected, it may be a method of discriminating fish spawned in areas such as the Coral Sea, from more tropical spawning locations in the Western Pacific.

4.2 VARIATION IN ALLOZYMES AND MITOCHONDRIAL DNA

A full account of the genetics component of the stock delineation research is provided in Appendix 2, which is the manuscript of a paper in press (Ward et al., 1994) in *Marine Biology*. Samples of yellowfin tuna from the western, central and eastern regions of the Pacific Ocean (Table 1) were examined for genetic variability. A summary of this work follows, while Appendix 2 should be consulted for further details. (Note that in the Appendix, *GPI-1** is referred to as *GPI-F**, and *GPI-2 *as GPI-S**)

Comparison of all sites

An initial survey of 34 allozyme loci in about 20 individuals revealed five loci that were sufficiently polymorphic to provide useful data for genetic stock analysis. Four such loci (*ADA*, GPI-2*, GPI-1** and *FH**), expressed in muscle tissue, were examined in all samples (n, number of fish, between 386 and 429 per locus) and a fifth polymorphism (*GDA**), expressed in liver tissue, examined in western and central Pacific samples only (n=179). This reduced sample size was because this protein had partially degraded in some of the samples we received, and could not then be unequivocally scored.

Samples (n=434) were also screened for mitochondrial DNA variation following restriction analysis by two enzymes (*BcI*I and *Eco*RI) detecting polymorphic cut sites. Eighteen mtDNA haplotypes were revealed, with an overall nucleon diversity of 0.678. A subset of animals (n=118) screened for eight restriction enzymes had an overall nucleon diversity of 0.724 and a mean nucleotide diversity per sample of 0.359%.

No significant spatial heterogeneity was detected for alleles at the *ADA*, GDA*, GPI-2** and *FH** loci . This was the first time that these loci had been used in the genetic analysis of yellowfin populations. Significant heterogeneity was detected for *GPI-1**, a locus earlier examined by Sharp (1978). At this locus, the two eastern samples (southern California and northern Mexico) were not significantly different from each other but were significantly different ($P<0.001$) from the five western/central samples (Coral Sea, Philippines, Kiribati, Hawaii-91 and Hawaii-92), which as a group showed no significant heterogeneity. *GPI-1*100* was the most common allele in western and central regions, *GPI-1*75* the most common in eastern samples. This result confirmed and extended Sharp's (1978) data, which had earlier shown that a GPI polymorphism did significantly differentiate two eastern Pacific samples from one in the western Pacific.

No significant heterogeneity was detected for the mtDNA haplotypes. After our paper had gone to press in *Marine Biology*, we learnt of a similar, if less intensive, study of mtDNA in Pacific populations of yellowfin tuna carried out by Scoles and Graves (1993). These authors had looked at five Pacific samples (New South Wales, Papua New Guinea, Hawaii, Mexico, Ecuador) for mtDNA variation alone, with samples of 20 per site (total n=100). This compares with seven sites and mtDNA sample sizes per site of between 34 and 97 (total n=434) in our study. They similarly found no mtDNA population differentiation, and concluded that the

null hypothesis that yellowfin tuna in the Pacific share a common gene pool could not be rejected.

Our data indicate that yellowfin tuna in the Pacific do *not* share a common gene pool, and that *GPI-1** variation defines two reproductively isolated groups in the Pacific. One of these occupies western and central regions, while the other occupies the eastern seaboard. This broad-scale spatial heterogeneity offers some hope that with an increased sample effort more fine-scale heterogeneity might be uncovered. This increased sample effort should include not only increasing sample size but also increasing the sampling intensity at the DNA level.

Comparison of the two juvenile samples.

Two of the samples, Hawaii 1991 and the Philippines, were of small fish between 3 and 6 months of age. These juveniles are likely to still be in the vicinity of their spawning grounds while all other samples were of larger fish which could now be a considerable distance from their natal ground and could therefore comprise mixtures from different natal areas. It was therefore of interest to compare the genetic make-up of the Hawaii91 and Philippines samples, since if there were to be any genetic differentiation among our western/central Pacific samples, it should be between these two well-separated collections of juveniles. This comparison was not considered in the *Marine Biology* manuscript, which looked only at the broad question of Pacific-wide differentiation of yellowfin tuna.

Allele frequencies and mtDNA haplotype frequencies (data given in Appendix 2) were compared between the two samples using the Roff and Bentzen procedure (see Appendix 2). Probabilities of no significant differentiation, based on 1000 Monte-Carlo simulations per comparison, were: *ADA** (χ^2=1.388, P=0.508), *FH** (χ^2=4.672, P=0.069), *GPI-2** (χ^2=2.876, P=0.187), *GPI-1** (χ^2=6.205, P=0.079), *GDA** (χ^2=8.873, P=0.040) and mtDNA (χ^2=6.065, P=0.449). Three of the six comparisons had probabilities below 0.10, with one of these having a probability just below 0.05. While there are clearly no large differences in frequencies between the two samples , there is some weak evidence of genetic differentiation. This needs to be confirmed, or refuted, from larger samples. If it turns out that larval or juvenile yellowfin show evidence of genetic spatial heterogeneity, while high-seas or adult tuna do not, this would constitute evidence for adult admixture followed by site-fidelity. If, on the other hand, larval, juvenile and adult yellowfin show spatial heterogeneity, then this would constitute evidence for limited migration away from natal areas.

Prospects for further work

In order to delineate further the genetic structure of yellowfin stocks in the western Pacific, and in particular to define the origin of recruits to the east coast yellowfin tuna fishery, two things need to be done:

- 1.   Sample sizes need to be larger than those of this pilot study. It is clear that genetic differences among samples from the western Pacific are likely to be limited, due to gene flow among areas, and small samples will be insufficient to detect the small genetic

differences that may be present. Target sample sizes of 200 from each of several Coral Sea rim areas are suggested, with a larger sampling effort off New South Wales.

- 2.   These samples should be analysed not only for allozymes and mtDNA polymorphisms, using methodologies developed in this pilot study, but also for non-coding nuclear DNA, using newly-developed techniques.

Recent advances in genetic technologies now make it possible to screen for variation in sequences of non-coding nuclear DNA. For stock delineation, such markers will have several advantages over both allozyme and mtDNA markers. Non-coding DNA, sometimes referred to as "junk" DNA, has mutation rates several orders of magnitude greater than allozymes or mtDNA, meaning that populations will differentiate not only by drift but also by mutation. This should provide a more powerful method of stock discrimination. In addition, non-coding sequences will not be affected by natural selection, while natural selection on coding sequences may either enhance or diminish population differences. Thus data from non-coding sequences can be less ambiguously interpreted.

There have been, so far, few studies published examining these new classes of non-coding DNA, one class of which comprises the so-called mini and micro-satellite loci, while another class consists of non-coding sequences flanking coding genes. However, a preliminary survey of trout (*Salmo trutta*) microsatellites concluded that "it is likely that microsatellite variation will magnify the differentiation observed in enzyme surveys" (Estoup et al., 1993). Non-coding DNA regions of both American oysters (*Crassostrea virginica*) and cod (*Gadus morhua*) showed much greater levels of population differentiation than was apparent following earlier allozyme analysis (Karl and Avise, 1992; Pogson et al., 1994). In both cases, the authors concluded that gene flow among areas is more restricted than was previously thought from allozyme studies, and that the allozyme similarities were due to balancing selection for the same genotypes in different localities rather than to extensive gene flow. The conclusion of balancing selection on allozymes could be premature, since it is conceivable that differences in mutation rate were responsible: however, the major, and very exciting finding as far as stock delineation studies are concerned, is that both studies showed that the analysis of non-coding DNA is a much more powerful population discriminator than allozyme analysis.

Examining yellowfin samples for variation in these non-coding sequences of DNA is likely to provide the single most powerful genetic discriminator of populations, although still greater power will be achieved by the simultaneous use of multiple classes of genetic markers: non-coding DNA, allozymes, and mtDNA haplotypes. We suggest that any follow-up genetic study should study all three types of marker.

4.3 GENETIC IDENTIFICATION OF THUNNUS SPECIES

Methods for identifying commercially important species and larvae have traditionally relied on morphological characters. Such characters can be unreliable as they may be environmentally influenced or, in the case of commercial specimens, can sometimes be intentionally removed. Genetic characters cannot be removed, and are not ontogenetically influenced by environmental conditions during the growth of a fish: they are therefore more reliable for species identifications.

The extent of genetic differentiation between the six major tuna species found in Australian waters was assessed using both allozyme and mitochondrial DNA methods. The species examined were:

YF	Yellowfin	*Thunnus albacares*
SBT	Southern Bluefin	*Thunnus maccoyii*
NBT	Northern Bluefin	*Thunnus thynnus orientalis*
BE	Bigeye	*Thunnus obesus*
ALB	Albacore	*Thunnus alalunga*
SKJ	Skipjack	*Katsuwonus pelamis*

Sample sizes were highly variable for the different species. Genetic markers shown to be polymorphic at appreciable frequencies in yellowfin and southern bluefin tuna were examined in large numbers as part of stock delineation work, while other loci were typically examined in much smaller numbers: the allozyme sample numbers reflect this variation. In the mitochondrial DNA analysis, data from only those fish examined for all 15 restriction enzymes are presented. Locations of these fish are given in Table 9.

Samples of white muscle and liver were obtained for all species except the northern bluefin, where only muscle samples were obtained. Tissues were generally taken on board vessels, frozen at -20C and air-freighted frozen to the laboratory where they were held at -80C until extracts were prepared for analysis.

Allozyme analysis

Small pieces of liver or muscle tissue were placed in 1.5 ml microcentrifuge tubes, homogenized manually with a few drops of distilled water, and spun at 10 000 rpm in a micro-centrifuge for 2 minutes. The supernatant was used for electrophoresis, which was carried out using two gel systems. Gel system A used Helena Titan III cellulose acetate plates run with a tris-glycine buffer (0.02M tris, 0.192M glycine; see Hebert and Beaton (1989) for details), and gel system B used 12% Sigma starch gels and a histidine/citrate buffer system (gel buffer: 0.005M histidine HCl, adjusted to pH 7.0 with 0.1M sodium hydroxide; electrode buffer: 0.41M trisodium citrate, adjusted to pH 7.0 with 0.5M citric acid).

Table 9. Species, sample size (n), location, collector, and date collected of the tuna specimens used in the mtDNA component of the taxonomic study. Additional samples, especially of yellowfin and southern bluefin, were examined for some of the allozyme loci.

Species	n	location	collector	Date
Albacore	3	Hawaii, USA	P. Grewe	Aug. 91
	6	Tasmania, Australia	N. Harper	May 92
Bigeye	7	Hawaii, USA	P. Grewe	Aug. 91
Northern bluefin	1	California, U.S.A.		Aug. 92
	1	Tasmania, Australia	RTMP Obs.	Jan. 93
	1	Fish Market, Japan	T. Polacheck	Oct. 92
	4	NRIFSF, Japan	S. Chow	Oct. 92
Skipjack	3	Tasmania, Australia	N. Harper	May 92
Southern bluefin	13	South Africa	RTMP	Oct. 92
	13	Tasmania	RTMP	June 92
Yellowfin	4	Coral Sea	J. Gunn	Nov. 91
	4	Philippines	T. Davis	March 91
	12	Hawaii, USA	C. Boggs	Oct. 91

Table 10 presents details of the 23 enzyme systems, representing a minimum of 37 loci, that were analysed. Liver samples were not available for the northern bluefin tuna, and as a consequence only 26 loci could be screened in this species.

Loci and alleles were designated following the nomenclature system recommended by Shaklee et al. (1990). Multiple loci encoding the same enzyme were designated by consecutive numbers, with '1' denoting the fastest migrating system. Alleles within each locus were numbered according to the anodal mobility of their product relative to that of the common allele observed in the yellowfin tuna, which was designated '100'. Cathodal migration of enzyme product was designated by a negative number. Note that phenotypes rather than genotypes were ascribed to the *PEPA**, *PEPB** and PROT patterns (Fig. 9), and these loci were not used in the genetic distance calculations. This was because of uncertainties in determining band homologies across species.

Table 10. Enzyme systems employed in the allozyme taxonomy. Tissue: l - liver, m - muscle, e - eye. Gel system: A - cellulose acetate, B - starch (see text).

Enzyme System	Locus Abbrev.	Locus No.	EC Number	Tissue	Gel
Aspartate aminotransferase	*mAAT**	1	2.6.1.1	m	A
	*sAAT-1**	2		m	A
	*sAAT-2**	3		l	A
Adenosine deaminase	*ADA**	4	3.5.4.4	l	A
Alcohol dehydrogenase	*ADH**	5	1.1.1.1	l	A
Aconitate hydratase	*sAH**	6	4.2.1.3	l	A
Aldolase	*ALD-1**	7	4.1.2.13	e	A
	*ALD-2**	8		l/m	A
Creatine kinase	*CK-1**	9	2.7.3.2	e	A
	*CK-3**	10		m	A
Esterase-D (UV, umb acetate)	*ESTD-2**	11	3.1.-.-	m/l	A/B
Fumarate hydratase	*FH**	12	4.2.1.2	m	A
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH-1**	13	1.2.1.12	m	A
	*GAPDH-2**	14		m	A
Guanine deaminase	*GDA**	15	3.5.4.3	l	A
Glucose-6-phosphate isomerase	*GPI-1**	16	5.3.1.9	m	A
	*GPI-2**	17		m	A
Glycerol-3-phosphate dehydrogenase	*G3PDH-1**	18	1.1.1.8	l	A/B
	G3PDH-2•	19		m	A/B
L-Iditol dehydrogenase	*IDDH**	20	1.1.1.14	l	A
Isocitrate dehydrogenase	*mIDHP**	21	1.1.1.42	l	B
	*sIDHP**	22		m	B
L-Lactate dehydrogenase	*LDH-1**	23	1.1.1.27	e	A/B
	*LDH-2**	24		m	A/B
	*LDH-3**	25		m	A/B
Malate dehydrogenase	*mMDH-1**	26	1.1.1.37	m	A
	*mMDH-2**	27		m	A
Malic enzyme	*sMEP-1**	28	1.1.1.40	m	A
	*sMEP-2**	29		m	A
Mannose-6-phosphate isomerase	*MPI**	30	5.3.1.8	l	A
Peptidase - Glycyl-L-Leucine	*PEPA**	31	3.4.-.-	m	A
L-Phenylalanyl-L-Proline	*PEPB**	32		m	A
Phosphogluconate dehydrogenase	*PGDH**	33	1.1.1.44	m	B
Phosphoglucomutase	*PGM-1**	34	5.4.2.2	l	A
	*PGM-2**	35		l/m	A
General protein	*PROT*	36		m	A
Superoxide dismutase	*sSOD**	37	1.15.1.1	l	A/B

Fig. 9. Diagrammatic representation of the patterns observed for the peptidase and general protein stains on cellulose acetate plates.

Table 11 gives a list of allele frequencies and sample sizes. Table 12 presents two matrices of genetic distances (D) and identities (I) between pairs of species, using Nei's (1978) unbiased measures. The upper matrix gives D and I values for the 23 loci examined in all six species, the lower matrix gives values for the 34 loci examined in all but the northern bluefin tuna. *PEPA*, PEPB** and PROT are excluded from these analyses. These two matrices are converted into UPGMA-derived dendrograms in Figure 10.

The five *Thunnus* species are genetically very similar to one another, with genetic identities within the range 0.808 to 0.990 (mean of 0.905, based on 23 loci). Indeed, they are genetically much more similar than are most congeneric fish species, about 90% of which have genetic identities falling within the range of 0.4 to 0.8 (Thorpe, 1983). The species in the genus *Thunnus* appear to be of relatively recent origin. Not surprisingly, the skipjack tuna, located in a different genus from the other tunas considered here, is the most divergent species.

A dichotomous allozyme key enabling the identification of commercial tunas found in Australian waters follows. This is based on the electrophoresis of enzymes found in white muscle extracts, using the cellulose acetate system described above. Note that glycerol-3-phosphate dehydrogenase (G3PDH) has already been identified as an important taxonomic discriminator for tunas (Graves et al., 1988). Some additional discriminators between species may be found in Table 13. Skipjack, albacore and bigeye are very easy to identify, while yellowfin, southern bluefin and northern bluefin are more difficult to distinguish. The control referred to in the key is an extract of confirmed southern bluefin tuna.

1. CK-3 (the major muscle CK) slower than control (about 0.5x speed)...skipjack
   CK-3 equal in mobility to control ... go to 2

2. mAAT (the cathodal AAT) slower than control (about 0.5x speed) ... albacore
   mAAT equal to control ... go to 3.

3. G3PDH-2 (muscle G3PDH) faster than control (about 2x speed) ... bigeye
   G3PDH-2 equal to control ... go to 4

4. PEP-B pattern different from control (see Fig 9) ... yellowfin
   PEP-B pattern same as control ... go to 5.

5. ADA single fast band (see below) ... northern bluefin
   ADA same as control or with one band in common with control ... southern bluefin

ADA patterns:

NBT ------------SBT-------------------
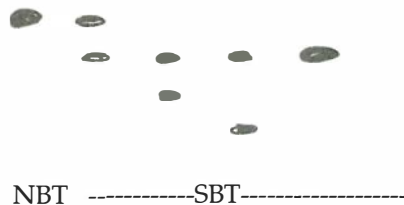
Note: a single fast ADA band is expected to be present in 1-2% of southern bluefin. Northern bluefin and southern bluefin separation can be checked by testing for PEP-A (see Fig 9).
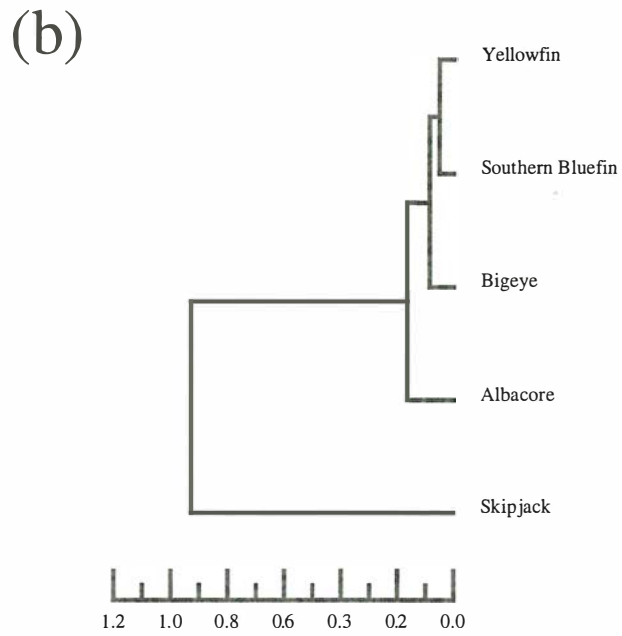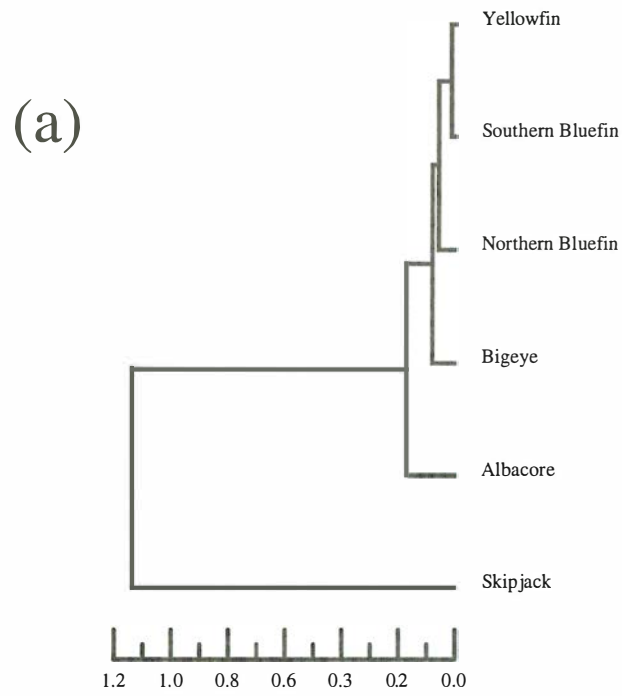
Figure 10. UPGMA -derived dendrograms of genetic distance (Nei, 1978).
(a) six species and 23 loci, (b) five species and 34 loci.

Table 11. Electrophoretic mobilities of each locus relative to the most common allele recorded from yellowfin tuna, Thunnus albacares. Locus details are presented in Table 10 and species abbreviations are presented in the text. Alleles are presented both as relative mobilities, and as letters, n: number of individuals scored, -: allele not detected, ns: locus not scored.

	Locus	Allele/n	YF	SBT	NBT	BE	ALB	SKJ
1	mAAT*	-55 (a)	-	-	-	-	1.000	-
		-100 (b)	1.000	1.000	1.000	1.000	-	1.000
		n	20	60	32	11	7	12
2	sAAT-1*	140 (a)	0.025	0.008	0.016	-	-	0.577
		100 (b)	0.975	0.992	0.984	1.000	1.000	0.423
		n	20	60	32	10	7	13
3	sAAT-2*	100 (a)	1.000	1.000		1.000	1.000	1.000
		n	14	7	ns	9	8	7
4	ADA*	145 (a)	-	-	-	-	0.500	-
		130 (b)	-	-	-	-	0.300	-
		115 (c)	0.355	0.113	1.000	1.000	0.100	-
		105 (d)	-	-	-	-	-	0.125
		100 (e)	0.609	0.832	-	-	0.100	-
		95 (f)	-	-	-	-	-	0.875
		85 (g)	0.035	0.052	-	-	-	-
		70 (h)	-	0.003	-	-	-	-
		n	411	580	32	16	5	12
5	ADH*	-50 (a)	-	-		-	-	0.143
		-80 (b)	-	-		-	-	0.857
		-100 (c)	1.000	1.000		1.000	1.000	-
		n	22	40	ns	8	4	7
6	sAH*	130 (a)	-	0.008		-	-	-
		115 (b)	0.094	0.992		-	0.938	0.083
		100 (c)	0.906	-		0.667	-	0.917
		90 (d)	-	-		0.333	0.062	-
		n	16	59	ns	9	8	6
7	ALD-1*	100 (a)	1.000	1.000		1.000	1.000	1.000
		n	7	5	ns	5	5	6
8	ALD-2*	100 (a)	-	-	-	-	-	1.000
		-100 (b)	1.000	1.000	1.000	1.000	1.000	-
		n	11	30	16	5	5	8
9	CK-1*	100 (a)	1.000	1.000		1.000	1.000	1.000
		n	12	5	ns	5	5	6
10	CK-3*	100 (a)	1.000	1.000	1.000	1.000	1.000	-
		60 (b)	-	-	-	-	-	1.000
		n	19	60	10	8	7	12
11	ESTD-2*	100 (a)	1.000	1.000	1.000	0.962	0.938	1.000
		75 (b)	-	-	-	0.038	0.063	-
		n	22	59	20	13	8	11

Table 11, continued

Locus	Allele/n	YF	SBT	NBT	BE	ALB	SKJ
12 *FH**	120 (a)	0.090	-	0.548	0.269	1.000	1.000
	100 (b)	0.903	1.000	0.452	0.731	-	-
	70 (c)	0.008	-	-	-	-	-
	n	390	59	21	13	7	12
13 *GAPDH-1**	100 (a)	1.000	1.000	1.000	1.000	1.000	1.000
	n	21	57	19	8	7	9
14 *GAPDH-2**	50 (a)	-	-	-	-	-	1.000
	-100 (b)	1.000	1.000	1.000	1.000	1.000	-
	n	16	55	2	7	8	11
15 *GDA**	145 (a)	-	-		-	0.313	-
	120 (b)	0.124	0.001		-	0.688	-
	110 (c)	0.331	0.454		-	-	-
	100 (d)	0.506	0.042		0.542	-	-
	90 (e)	0.037	0.417		0.458	-	0.278
	80 (f)	0.003	0.071		-	-	0.667
	70 (g)	-	0.015		-	-	0.056
	n	177	536	ns	12	8	9
16 *GPI-1**	130 (a)	0.038	-	-	-	-	-
	100 (b)	0.569	0.513	0.015	0.588	0.625	1.000
	80 (c)	0.392	0.481	0.970	0.412	0.375	-
	45 (d)	0.001	0.006	0.015	-	-	-
	n	430	578	32	17	8	12
17 *GPI-2**	-20 (a)	0.001	-	-	-	-	-
	-60 (b)	0.176	-	0.016	-	-	-
	-100 (c)	0.821	1.000	0.984	1.000	1.000	1.000
	-125 (d)	0.001	-	-	-	-	-
	n	433	578	32	16	8	12
18 *G3PDH-1**	150 (a)	-	-		-	-	1.000
	100 (b)	1.000	1.000		1.000	1.000	-
	n	17	31	ns	10	5	6
19 *G3PDH-2**	240 (a)	-	-	-	1.000	-	-
	120 (b)	-	-	-	-	-	1.000
	100 (c)	1.000	1.000	1.000	-	1.000	-
	n	24	59	20	18	5	12

Table 11, continued

	Locus	Allele/n	YF	SBT	NBT	BE	ALB	SKJ
20	IDDH*	260 (a)	0.025	0.018		-	-	-
		100 (b)	0.975	0.982		1.000	1.000	-
		20 (c)	-	-		-	-	1.000
		n	20	56	ns	7	8	8
21	mIDHP*	100 (a)	1.000	1.000	1.000	1.000	1.000	-
		-80 (b)	-	-	-	-	-	1.000
		n	22	60	33	11	7	10
22	sIDHP*	100 (a)	1.000	1.000	1.000	1.000	1.000	-
		75 (b)	-	-	-	-	-	1.000
		n	21	8	24	8	6	10
23	LDH-1*	100 (a)	1.000	1.000		1.000	1.000	1.000
		n	12	5	ns	5	5	6
24	LDH-2*	100 (a)	1.000	1.000	1.000	1.000	1.000	1.000
		n	19	60	10	9	7	11
25	LDH-3*	-50 (a)	-	-	-	-	-	1.000
		-100 (b)	1.000	1.000	1.000	1.000	1.000	-
		n	19	60	10	9	7	11
26	sMDH-1*	100 (a)	1.000	1.000	1.000	1.000	1.000	-
		70 (b)	-	-	-	-	-	1.000
		n	13	30	31	6	5	11
27	sMDH-2*	100 (a)	1.000	1.000	1.000	1.000	1.000	-
		10 (b)	-	-	-	-	-	1.000
		n	22	39	33	8	7	11
28	sMEP-1*	100 (a)	1.000	1.000	1.000	0.958	1.000	-
		95 (b)	-	-	-	-	-	1.000
		80 (c)	-	-	-	0.042	-	-
		n	23	40	26	12	8	13
29	sMEP-2*	125 (a)	-	-	-	0.042	-	-
		100 (b)	1.000	1.000	1.000	0.958	1.000	-
		80 (c)	-	-	-	-	-	1.000
		n	23	41	26	12	8	13
30	MPI*	120 (a)	-	0.018	0.047	-	-	-
		100 (b)	1.000	0.974	0.953	1.000	1.000	1.000
		85 (c)	-	0.006	-	-	-	-
		65 (d)	-	0.003	-	-	-	-
		n	18	571	32	10	7	12
31	[1]PEPA*	-		A	B	A	A	C	D
		n	23	23	33	5	4	5
32	[1]PEPB*	-		A	B	B	A	B	C
		n	32	30	33	5	4	7
33	PGDH*	180 (a)	-	-	-	-	-	1.000
		125 (b)	-	0.003	-	-	-	-
		100 (c)	1.000	0.703	1.000	1.000	1.000	-
		75 (d)	-	0.276	-	-	-	-
		50 (e)	-	0.019	-	-	-	-
		n	22	567	33	11	6	12

Table 11, continued

Locus	Allele/n	YF	SBT	NBT	BE	ALB	SKJ
34 *PGM-1**	110 (a)	–	0.006		–	–	–
	100 (b)	1.000	0.420		0.278	0.063	–
	90 (c)	–	0.557		0.722	0.938	–
	80 (d)	–	0.016		–	–	–
	75 (e)	–	–		–	–	0.111
	70 (f)	–	0.001		–	–	0.889
	n	17	534	ns	9	8	9
35 *PGM-2**	200 (a)	–	0.001	–	–	–	–
	100 (b)	1.000	0.999	1.000	1.000	–	0.038
	50 (c)	–	–	–	–	0.563	0.962
	-50 (d)	–	–	–	–	0.438	–
	n	23	538	26	14	8	13
36 [1]*PROT*	–	A	A	A	B	B	C
	n	30	22	33	12	7	12
37 *sSOD**	100 (a)	1.000	1.000		1.000	1.000	1.000
	n	13	54	ns	6	5	5

[1]General Protein and Peptidases presented as patterns observed, rather than alleles

Table 12. Estimates of genetic identity (below diagonal) and genetic identity (above diagonal) (Nei, 1978) between pairs of species, based on allozyme allele frequencies.

(a) All six species and 23 loci

	YF	SBT	BE	ALB	NBT	SKJ
YF	*	0.010	0.067	0.145	0.044	1.145
SBT	0.990	*	0.089	0.166	0.065	1.170
BE	0.935	0.915	*	0.213	0.073	1.145
ALB	0.865	0.847	0.808	*	0.139	1.057
NBT	0.957	0.937	0.930	0.870	*	1.161
SKJ	0.318	0.310	0.318	0.348	0.313	*

(b) Five species (NBT excluded) and 34 loci

	YF	SBT	BE	ALB	SKJ
YF	*	0.049	0.070	0.168	0.911
SBT	0.952	*	0.093	0.129	0.957
BE	0.932	0.911	*	0.187	0.913
ALB	0.845	0.879	0.829	*	0.923
SKJ	0.402	0.384	0.401	0.397	*

Table 13. Allele presence at each of 37 loci for each of the six species examined. Locus numbers and alleles are shown in Table 11
- denotes that the locus was not scored.

Spp.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
YF	b	ab	a	ceg	c	bc	a	b	a	a	a	abc	a	b	bcdef	abcd	abcd	b	c	ab	a	a	a	a	b	a	a	a	b	b	a	a	c	b	b	a	a
SBT	b	ab	a	cegh	c	ab	a	b	a	a	a	b	a	b	bcdefg	bcd	c	b	c	ab	a	a	a	a	b	a	a	a	b	abcd	b	b	bcde	abcdf	ab	a	a
NBT	b	ab	-	c	-	-	-	b	-	a	a	ab	a	b	-	bcd	bc	-	c	-	a	a	-	a	b	a	a	a	b	ab	a	b	c	-	b	a	-
BE	b	b	a	c	c	cd	a	b	a	a	ab	ab	a	b	de	bc	c	b	a	b	a	a	a	a	b	a	a	ac	ab	b	a	a	c	bc	b	b	a
ALB	a	b	a	abce	c	bd	a	b	a	a	ab	a	a	b	ab	bc	c	b	c	b	a	a	a	a	b	a	a	a	b	b	c	b	c	bc	cd	b	a
SKJ	b	ab	a	df	ab	bc	a	a	a	b	a	a	a	a	efg	b	c	a	b	c	b	b	a	a	a	b	b	b	c	b	d	c	a	ef	bc	c	a

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Mitochondrial DNA

Although the project proposal did not call for an assessment of the suitability of mitochondrial DNA (mtDNA) analysis for tuna identification, such an assessment was carried out.

Total DNA was extracted from white muscle tissue using a modified CTAB (hexadecyltrimethylammoniumbromide) protocol described by Grewe et al. (1993).

Individuals were screened with 15 restriction enzymes (*Ava*I, *Bam*HI, *Ban*I, *Bcl*I, *Dra*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Nco*I, *Nde*I, *Pst*I, *Pvu*II, *Sac*II, *Xba*I, and *Xho*I). Restriction fragments were separated following the electrophoresis procedure described by Grewe et al. (1993). Total DNA was transferred onto a nylon membrane filter (Hybond N+, Amersham Ltd) by southern transfer (Sambrook et al., 1989). The nylon membrane filters were probed with trevalla (*Hyperoglyphe antarctica*, Teleostei: Stromateoidei) mitochondrial DNA (50 ng used per 10 20cm x 20cm blots) purified by caesium chloride (CsCl) ultracentrifugation. The trevalla probe was labelled with [32]P dCTP (Bresatec Pty Ltd) using a GIGAprime DNA labelling kit (Bresatec Pty Ltd). The membrane filters were then exposed to Kodak XAR-5 X-ray film for 12-48 hours. Restriction fragments were given letters in the alphabetical order with which they were discovered. Haplotypes were given a three letter code corresponding to the species in which it found.

A total of 41 haplotypes was recognised among the fish examined with the 15 restriction enzymes (Table 14) For skipjack, 12 enzymes (*Ava*I, *Bam*HI, *Ban*I, *Bcl*I, *Dra*I, *Eco*RI, *Hin*dIII, *Nco*I, *Pvu*II, *Sst*II, *Xba*I, and *Xho*I) produced patterns not observed among the *Thunnus* species. Only *Dra*I produced unique patterns for each of the six species examined, although the small sample sizes together with the similarity in some of the *Dra*I patterns means that relying on this enzyme alone for species identification could lead to errors. The rest of the enzymes produced patterns which were shared among species. However, for each species pair a minimum of two enzymes produced patterns which were not shared between individuals of either species. From this group of 12 enzymes, *Sst*II and *Xho*I both failed to reveal any variant patterns among the five *Thunnus* species.

A minimum combination of four enzymes (*Bcl* I, *Dra*I, *Nco*I, and *Pst*I) could be used confidently to define haplotypes that were unique to each species. These enzymes plus three additional ones (*Ava* I, *Pvu* II, and *Sst*II) were used to define a dichotomous key which could be used as a guide to identify a muscle sample to species of origin (Figure 11).

The present study has demonstrated the potential of restriction analysis of mtDNA for the identification of commercially important tuna species. Given the technology of the present study, this identification can now be accomplished with a sample of muscle tissue as small as 10 mg. Future research should now be directed towards establishing the amount of

Table 14. mtDNA haplotypes observed among skipjack (SKJ-group), albacore (ALB-group), northern bluefin (NBT-group), bigeye (BE-group), southern bluefin (SBT-group), and yellowfin tuna (YF-group) . n: number of individuals scored

Hapl. group	n	Ava I	Bam HI	Ban I	Bcl I	Dra I	Eco RI	Hind III	Kpn I	Nco I	Nde I	Pst I	Pvu II	Sst II	Xba I	Xho I
SKJ-1	1	C	C	E	K	I	E	C	A	D	O	C	C	B	C	B
SKJ-2	1	C	C	F	K	J	D	C	A	D	O	C	C	B	C	B
SKJ-3	1	C	C	E	K	J	D	C	A	D	O	C	C	B	C	B
ALB-1	2	A	A	C	G	L	A	A	B	B	A	G	B	A	G	A
ALB-2	2	A	A	C	G	L	A	A	B	B	A	D	B	A	B	A
ALB-3	1	A	A	B	G	L	A	A	B	B	A	C	B	A	B	A
ALB-4	1	A	A	C	G	L	A	A	B	B	A	D	B	A	G	A
ALB-5	1	A	A	C	I	L	A	A	B	B	A	D	B	A	B	A
ALB-6	1	A	A	C	J	L	A	A	B	B	A	C	B	A	B	A
ALB-7	1	A	A	C	G	L	A	A	B	B	A	G	B	A	H	A
NBT-1	2	A	A	C	G	E	A	A	B	A	A	E	B	A	G	A
NBT-2	1	A	A	C	G	E	A	A	B	B	A	E	B	A	B	A
NBT-3	1	A	A	C	G	E	A	A	C	A	A	E	B	A	B	A
NBT-4	1	A	A	C	G	E	A	A	B	B	A	E	B	A	G	A
NBT-5	1	A	A	C	H	E	A	A	B	B	A	E	B	A	G	A
NBT-6	1	A	A	C	G	E	A	A	B	B	A	F	B	A	B	A
BE-1	3	A	A	A	H	G	A	A	A	C	C	A	A	A	I	A
BE-2	1	A	A	A	H	G	A	A	A	A	O	A	A	A	I	A
BE-3	1	B	A	A	G	G	A	B	A	A	B	A	A	A	A	A
BE-4	1	A	A	A	H	G	A	A	A	C	B	A	A	A	I	A
BE-5	1	A	A	A	H	G	F	A	A	C	B	A	A	A	A	A
SBT-1	12	A	B	A	F	A	A	A	B	A	A	B	A	A	A	A
SBT-2	7	A	A	A	F	A	A	A	B	A	A	B	A	A	A	A
SBT-3	2	A	A	A	F	A	B	A	B	A	A	B	A	A	A	A
SBT-4	1	A	B	A	F	A	B	A	B	A	A	B	A	A	A	A
SBT-5	1	B	B	A	F	A	A	A	B	A	A	B	A	A	A	A
SBT-6	1	A	B	A	A	B	A	A	B	A	A	B	A	A	A	A
SBT-7	1	A	A	A	F	B	A	A	B	A	A	B	A	A	A	A
SBT-8	1	A	A	A	R	A	A	A	B	A	A	B	A	A	A	A
YF-1	7	A	A	A	A	F	B	A	A	C	A	A	A	A	A	A
YF-2	4	A	A	A	A	F	A	A	A	C	A	A	A	A	A	A
YF-3	1	A	A	A	B	F	A	A	A	C	A	A	A	A	D	A
YF-4	1	A	A	A	A	F	B	A	A	C	A	B	A	A	A	A
YF-5	1	A	A	A	C	F	B	A	A	C	A	A	A	A	A	A
YF-6	1	A	A	A	B	F	B	A	A	C	A	A	A	A	A	A
YF-7	1	A	A	B	A	F	B	A	A	C	A	A	A	A	A	A
YF-8	1	A	A	A	C	F	A	A	A	C	A	A	A	A	A	A
YF-9	1	A	B	A	A	F	B	A	A	C	A	A	A	A	A	A
YF-10	1	A	A	A	A	F	B	A	A	C	B	A	A	A	A	A
YF-11	1	A	A	A	A	F	A	A	B	C	A	A	A	A	A	A
YF-12	1	A	A	A	A	F	A	A	A	C	A	A	B	A	A	A

Figure 11. Dichotomous key defining restriction enzymes used to separate and identify species of tuna. Letters in parenthesis indicate restriction profile used to define a specific tuna lineage. Note that this diagram/key should not be read as indicating evolutionary relationships, as it is based only on a non-random set of restriction enzymes useful in species recognition.

AvaI (C),
SstII (B)

Skipjack

DraI (L),
PstI (C,D,G)

Albacore

PvuII (B)

BclI
(G,H,I,J)

DraI (E),
PstI (E,F)

Northern Bluefin

AvaI (A,B),
SstII (A)

PvuII (A)

Bigeye

DraI (A,B),
NcoI (A)

Southern Bluefin

BclI
(A,B,C,F,R)

DraI (F),
NcoI (C)

Yellowfin

intraspecific variation which exists among each of these closely related taxa. Quantitative assessment of intraspecific variability of these genetic markers will then establish the degree of accuracy and confidence which can be placed on identifications made using these genetic techniques.

Discussion

The ability to discriminate among the commercial species of Australian tuna using only a small piece of frozen white muscle is not in doubt. In fact, using these techniques, several examples of incorrectly identified tuna tissues were found among the samples sent to CSIRO for the yellowfin stock delineation research. Note though, that for some allozymes and mtDNA haplotypes, sample sizes are limited and the species-specificity of some of the patterns needs to be confirmed from larger samples.

One problem with allozyme analysis is that the tissue needs to have been frozen and kept frozen soon after the fish was captured. This is sometimes logistically difficult, and is not required for DNA analysis. Another disadvantage of the allozyme approach is that the *Thunnus* species are allozymically very closely related to one another, meaning that the allozyme characteristics separating the species are sometimes relatively minor (especially among the yellowfin, southern bluefin and northern bluefin tuna), while the mtDNA differences are more marked.

While either allozyme or mtDNA approaches can be used for species identification, we would recommend the mtDNA approach as the primary diagnostic tool, despite relatively minor drawbacks in cost and time. While not directly validated in our work on this project, it should prove possible to transport, at ambient temperatures, small pieces of muscle soaked in 90-95% alcohol and to analyse these pieces for mtDNA variation. This could be accomplished either by using a mtDNA probe, as in our present work, or by using the polymerase chain reaction (PCR) to amplify a specified region of mtDNA and then identifying the species that this mtDNA sequence came from.

Such an approach will be especially beneficial for larval identifications. While larval identification using the above allozyme key will be accurate if larval proteins are in good condition, proteins in larvae may degrade even when maintained frozen, and may therefore not be amenable to allozyme analysis. DNA is more stable and larvae may be stored in alcohol rather than freezers. Even if partially degraded, DNA can be amplified and analysed using PCR. Such analytical methods require a short period of development before they can be routinely used, but in principle offer significant advantages over allozyme determinations.

5. SYNTHESIS AND DISCUSSION OF RESULTS

The primary purposes of this pilot study were twofold: (1) to develop an allozyme-based key for tuna identification, and (2) to develop methods for the genetic and otolith microchemical examination of yellowfin tuna, to apply these methods to larval or juvenile tuna which could be assumed to be close to their spawning grounds. and to seek evidence of any spawning-site signals that could be used in a full-scale project aimed at identifying the source of recruits to the Australian east coast tuna fishery.

Tuna identifications

Keys were developed allowing the identification of the major Pacific tunas from white muscle samples. Both allozyme and mitochondrial DNA keys were developed, with the mtDNA approach having the advantage that transport of tissue can be in alcohol rather than frozen, but the disadvantage that protocols are more expensive and more time-consuming. CSIRO is now in the position to provide management agencies with an accurate identification of tunas based on muscle samples.

It is clear that the five *Thunnus* species examined are genetically closely-related to one another, and in evolutionary terms are likely to be recently derived. Nonetheless, diagnostic differences were found enabling species characterization. Indeed, the development of these keys benefited the stock delineation project as it identified a number of bigeye tuna specimens among the samples sent to us as yellowfin. These could not be identified from otolith chemical analysis, pointing to an incidental advantage of combining these two approaches in stock delineation studies.

Stock Delineation

Appropriate methodologies were developed for otolith microchemical analysis and for allozyme and mitochondrial DNA (mtDNA) analyses, but it proved impossible to collect larvae in sufficient numbers for meaningful examination. However, 3-6 month old juveniles were sampled from Hawaii (the 1991 collections - the 1992 collections were of 1+ group fish), Philippines, and Indonesia (only otoliths taken).

Significant differentiation in the micro chemical make-up of that portion of the otolith laid down during the first few days of larval life was observed among these three juvenile samples. This suggests that there may indeed be spawning-site signals in the larval or primordial regions of otoliths. Interestingly, the 1+ group fish from the Coral Sea could be differentiated from these three samples, suggesting that the Coral Sea fish were spawned in a fourth area, for example the Coral Sea itself. There was some overlap between the chemical signals of the samples, with misclassification rates of between 30 and 15%. At least with respect to the juvenile fish, these are unlikely to represent population admixture, but are rather a consequence of natural overlap in these signals among areas.

The greatest level of similarity was between the Hawaiian and Coral Sea samples, possibly reflecting the lower water temperatures of spawning and larval development of these fish compared with the Indonesian and Philippine fish. Otoliths are largely made up of calcium carbonate, and in carbonates the O18:O16 ratio is known to be related to deposition temperature: if differences in O18:O16 ratios can be detected, then this could be a method of discriminating fish spawned in areas such as the Coral Sea from more tropical locations.

If there are spawning-site signals in the otoliths, then these could be used to deduce the likely origins of adult fish. However, much work is required to corroborate this hypothesis. This includes, for example, examining more larvae/juveniles from more sites, and determining the magnitude of inter-annual variability in these signals. While the data from the pilot study were not, and were never intended to be, conclusive, they do offer substantial encouragement that such spawning signals do exist and can be utilised.

Genetic samples were not collected from the Indonesian fish, but the Hawaiian and Philippine juvenile fish did show some, albeit weak, evidence of genetic differentiation. However, fish from Kiribati, estimated to be about 1 year old, showed no evidence of genetic differentiation from the Philippine sample, despite being separated by about 6500 km. In fact, there was no clear genetic separation of fish from any of the central and western Pacific samples, although sample sizes were too small to detect any minor but biologically meaningful differences in gene frequency that may have existed. Yellowfin tagging carried out in this area shows that while most movements appear to be on relatively local longitudinal scale, there are also numerous records of yellowfin moving from sub-tropical to Japanese (temperate) waters (Lewis, 1992).Two major areas of high larval densities in the western/central equatorial Pacific have been described (130°E-170°E and 130°W-160°W), but spawning appears to be generally widespread (Suzuki et al., 1978). In this part of the Pacific, movements of a few hundred kilometres of most fish, taken together with the more extensive movements of a minority of fish and the presence of widespread spawning, probably provides sufficient gene flow to account for the apparent lack of genetic heterogeneity. In the face of this expected gene flow, and therefore low expected levels of genetic differentiation, sample sizes need to be increased in any full-scale study.

Genetic data were also collected on Mexican and Californian yellowfin, ie. on a pan-Pacific scale. The rationale here was that if no population differences could be detected on such a large scale, then it was unlikely that any differences would be detected on a more local scale. In fact, eastern Pacific yellowfin were found to be clearly differentiated from the western/central samples for the allozyme locus *GPI-1**, although not for three other polymorphic allozymes nor for mtDNA haplotypes. This finding shows that there must be at least two reproductively isolated groups of yellowfin in the Pacific, although catch data do not indicate any break in the distribution of yellowfin in the Pacific equatorial region (Suzuki et al., 1978; Lawson, 1992). Yellowfin larvae are found in higher density nearer islands (Leis et al., 1990, Lawson, 1992), and the relative absence of islands in the eastern Pacific and their abundance in the western/central region could account for the genetic isolation of fish of these two areas.

To summarise: this study found strong evidence of spawning-site specific chemical signals in yellowfin otoliths, and demonstrated the presence of at least two reproductively isolated stocks in the Pacific (western/central, and eastern). There were some suggestions of genetic differentiation of juveniles from western/central populations, but additional data are required to confirm or refute these suggestions. This pilot study offered considerable encouragement that a full-scale combined otolith and genetic programme, incorporating refinements to both components (such as O18:O16 analysis of otoliths, and examination of non-coding DNA sequences) would provide a much better understanding of yellowfin stock structure, and in association with tagging data should resolve the question of the origin of the Australian east coast yellowfin.

6. RECOMMENDATIONS

- 1. Routine genetic identification of tunas should be based on mitochondrial DNA analysis of white muscle, although if frozen white muscle is available, skipjack, albacore and bigeye can be more quickly and more cheaply identified by allozyme electrophoresis.

In order to determine more fully the stock structure of western /central Pacific yellowfin, and to determine the likely origin of recruits to the Australian east coast yellowfin fishery, the following recommendations are made:

- 2. A full-scale otolith and genetics study be carried out, building on the successes of the pilot-study in providing preliminary evidence of population heterogeneity. These two approaches provide complementary information, and their joint deployment provides a powerful tool for the resolution of stock structure issues.

- 3. Sample sizes per locality be increased over those of the pilot study, to c. 200/locality for genetic analysis and c. 100/locality for otolith analysis. In order to assess temporal variation, inter-annual comparisons on fine geographic scales are also recommended.

- 4. The examination of non-coding DNA sequences (which are expected to show more inter-locality variability than coding sequences), should be included in the genetics component as well as continued examination of allozyme and mitochondrial DNA variability.

- 5. The examination of $O18:O16$ ratios (which may provide indications of water temperature at spawning) should be included in the otolith component as well as continued examination of otolith microchemical variability.

7. ACKNOWLEDGEMENTS.

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APPENDIX 1..

Discriminating the Origins of Yellowfin Tuna
— A Consulting Report for John Gunn

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Abstract: In the application of discrimination analysis, it has been very often to see people include very large number of feature variables in their data set while number of observations is relatively small. The misclassification rate based on this type of training data set is usually very high. By analysing a real data set of yellowfin tuna with 18 feature variables and 111 observation for four populations, we show in this report that by reducing the number of feature variables, the misclassification rate can be substantially reduced. Two methods are used to reduce the number of feature variables. One is variable selection based on likelihood criterion or the canonical variate analysis. The other is to project the data to low dimension space by using the the canonical variate analysis.

Keyword:

Discriminating the Origins of Yellowfin Tuna
— A Consulting Report for John Gunn

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1. Introduction

Yellowfin Tuna fishery has been a commercially important fishery in the east coast of Australia for decades. The tuna appeared in the east coast are believed to be born in the tropical pacific. It is very useful for the fishery management to know where the tuna appeared in the east coast are from. There have been some well known birth ground in the tropical pacific. They are Hawaii, Philippine, Indonesia and Coral Sea. So, the first step forward would be to investigate if we can somehow discriminate the tuna from these known birth ground. A genetic study was carried out to find out if there are any genetic difference among the fish from these birth places. However, it turned up that there was no any genetic difference at all. Then, scientists from CSIRO Division of Fisheries led by John Gunn started to look at if there are any chemistry differences by examining the tuna otolith corresponding to the tuna's early life peorid. It is believed that the different environmental conditions among the birth grounds will leave different chemical marks on the young fish's otolith. Under the assumption that the fish will not be able to swim away from their birth place in their first 20 days of life, the segment of "tree rings" corresponding to the first 20 days in the "ear born" is used to test the content of certain chemical elements. The main objective of this research is to find out if each birth ground will produce an unique pattern in the contents of certain chemical elements on the tuna, due to its unique environmental reality.

In this report discrimination analysis is carried out on the data set to investigate if the tuna from the four birth ground can be classified. We show in this report that there does exists unique pattern of chemical elements for each of the four birth ground, which enable us to discriminate tuna from the four birth grounds. We demonstrate that by projecting the 18 dimension data set to 3 dimension space, a much lower misclassified rate can be achieved. This has far more reaching implications on discrimination analysis of some fishery data sets. It is very common in fishery research that large number of feature variables are measured whereas the number of observations is relatively small. The misclassification rate based on this type of training data set is usually very high. By analysing the yellowfin tuna data set which has 18 feature variables and 111 observation for four populations, we show

in this report that by reducing the number of feature variables via variable selection or data projection into lower dimension space, the misclassification rate can be substantially reduced. Two methods are used to reduce the number of feature variables. One is variable selection based on likelihood criterion or the canonical variate analysis. The other is to project the data to low dimension space by using the the canonical variate analysis.

2. The data set

The data set used in the analysis contains measurements of 18 chemical elements on 111 yellowfin tuna from the four birth grounds (groups), with 35 from Hawaii, 25 from Philippine, 32 from Coral Sea and 19 from Indonesia. More than 18 (?) chemical elements were measured for each tuna in its otolith. However, only 18 of them give measurements above detection limit. Thus, these 18 chemical elements are selected as the feature variables. They are NAM, SRM, KM, SḾ, CLM, CAM, FE, NI, RB, ZN, CAP, MN, CU, BR, SRP, SN, HG and PB77. Hence, there are 18 feature variables and 111 observations in the data set.

By plotting boxplots for each of the 18 feature variables of the four groups, some outliers are detected for some of the feature variables in the data set. Due to already small sample size, we can not simply delete the observations which contain some "outlier" feature components. Thus, we have to correct the value of the "outlier" components. A simple way of doing that is to replace a "outlier" with the mean or median of the feature component of the group. However, in order to model the variability of that feature component, we used one of the bootstrap type by replacing a "outlier" with a random generated data point from the reminding data in the feature component.

Due to high dimensionality of the data set, it is difficult to be presented in its original form. Table 1 contains the pairwise Mahalanobis distances among the four groups, which gives some indication of how far away among the four groups.

TABLE 1: Pairwise Mahalanobis distances among the four groups.

	Hawaii	Philippine	Coral Sea	Indonesia
Hawaii	0	15.26	4.09	4.78
Philippine	15.26	0	8.78	25.05
Coral Sea	4.09	8.78	0	10.22
Indonesia	4.79	25.05	10.225	0

We see from Table 1 that (1) Philippine is well away from the rest of the groups; (2) The Hawaii is very close to the Coral Sea and the Indonesia while Coral Sea is quite away from the Indonesia. Hence we avoid expect a large misclassification rate (abbreviated as error rate). will occur between the Hawaii and the Coral Sea and

the Indonesia respectively, and a small error rate associated with the Philippine.

It is obvious that the data set has the problem of "curse of dimension", that is the number of feature variables is too many compared with the number of observations available. To obtain a good discrimination result, we have to reduce the dimensionality of the data set. This is because the performance of a discrimination rule, which is measured by misclassification rates, fails to improve once the number of feature variables has reached a certain threshold. This is the so-called peaking phenomenon. To avoid the problem, we have to reduce the dimension of the feature vectors. There are two approaches which can lead us to dimension reduction. One is to select a subset of variables among the eighteen, which can efficiently separate the groups. By looking at the subset of variables, it can help us to understand the environmental mechanism which leave different biological marks in the young tuna's era bone. Furthermore, as measuring the all 18 variables is very time consuming and expensive, it is much economical to measure only a subset of variables and does not lose at discrimination power at the same time. The second approach of dimension reduction is to project the data into a low dimension space, and carry on the discrimination analysis there. As we shall show below, the two approaches are well connected via the canonical variate analysis (CVA). We present results from the both methods in Sections 4 and 5 respectively.

Before we close this section, we like to introduce some notations which will be used throughout this report. Suppose there are a finite number, say, g, of distinct groups denoted as $P_1, \cdots, P_g$. There are n entities of known population origin on which a set of, say, p, features has been measured for each of these entities. Let define $x_j^{(i)} = (x_{j1}^{(i)}, \cdots, x_{jp}^{(i)})^T$ as the feature vector which contains the p feature measurements for the j-th entity from the population G_i. And let n_i be the total number of entities known from G_i for $i = 1, \cdots, g$. Then $n = n_1 + \cdots + n_g$. We usually call the data set $\{x_1^{(i)}, \cdots, x_{n_i}^{(i)}\}_{i=1}^g$ as training data set. In our yellowfin data set, we have $g = 4$, $p = 18$ and $n = 111$ observations with $n_1 = 35$, $n_2 = 25$, $n_3 = 32$ and $n_4 = 19$.

3. Selecting important feature variables

In this section the canonical variate analysis (Rao 1973; Kshirsagar 1972) and the normal distribution based stepwise variables selection procedure are used to find out which feature variables contribute the most for the separation among the groups. These selected variables will shed some light on how the different environmental conditions lead to different patterns of chemical composition.

We start with the canonical variate analysis. A canonical variate analysis does not need the normality assumption for the group distribution. Let $\overline{x}_i = n_i^{-1} \sum_{j=1}^{n_i} x_j^{(i)}$ be the sample mean of the group G_i for $i = 1, \cdots g$ and $\overline{x} = g^{-1} \sum \overline{x}_i$ be the overall sample mean cross all groups. Then, the between-group sums of squares B and pooled within group sums of squares S are defined as

$$B = \frac{1}{g-1} \sum n_i (\overline{x}_i - \overline{x})(\overline{x}_i - \overline{x})^T \text{ and}$$

$$S = n^{-1} \sum_{i=1}^{g} \sum_{j=1}^{n_i} (x_j^{(i)} - \overline{x}_i)(x_j^{(i)} - \overline{x}_i)^T,$$

respectively. Let d be the minimum of p and $g - 1$. The sample canonical variate analysisfinds those linear combinations $\gamma_l^T x \ l = 1, \cdots d$ of the original p feature variables in x that successively maximize the ratio

$$\gamma^T B \gamma / \gamma^T S \gamma. \tag{3.2}$$

and satisfy that the γs are orthogonal to each other respective to S, that is $\gamma_l^T S \gamma_k = 0$ for $l \neq k$.

These $\gamma_1, \cdots, \gamma_d$ are the new set of coordinates which separate the groups more efficiently than the original data coordinates. For a given data value x of p dimension, we can transform it as $(\gamma_1^T x, \cdots, \gamma_d^T x)$ within a lower $d \leq p$ dimension space. We shall discuss more on this data transformation in the next section related to dimension reduction. From a variable selection point of view, a feature variable is regarded as important if it corresponds to a larger (in absolute value) value in the canonical variates $\gamma_1, \cdots, \gamma_d$.

For the yellowfin data, $d = \min\{18, 4 - 1\} = 3$. We use SAS's CANDISC procedure (SAS Institute, 1990, p.387ff) to obtain the following canonical variates $\gamma_1, \gamma_2, \gamma_3$ listed in Table 2. By looking at the absolute values of the coefficients listed in the table, we see that Nam should be the first the be included as it has the largest coefficient value 1.606 at γ_1. Followed by Zn (0.806 at γ_2), KM (-0.778 at γ_1), SRM (0.601 at γ_1 and 0.491 at γ_3), CLM (-0.650 at γ_1), CAM (-0.558 at γ_1), CAP (0.466 at γ_2). The analysis reveals that the above seven variables play more important role in separating the four groups than rest of the feature variables and should be considered in the interpreting of the environmental conditions.

To confirm the above results from the canonical variate analysis, a Normal distribution based stepwise procedure is also used to select a subset of feature variables to produce a good discrimination results. The stepwise procedure uses the forward selection and backward elimination of the feature variables. It assumes the population groups are multivariate normal distributed with a common covariance matrix. Figure 1 gives QQ plots of the Mahalanobis distance for each group. The Mahalanobis distance are the distance between each data point in a group and the center of that group. Figure 1 exhibit basically a linear shape in the QQ plots, which implies the normal assumption is reasonable. The stepwise procedure selects or dismisses a variable according to the significance level of an F test from an analysis of covariance, where the variables already selected act as covariates and the variable under consideration is the dependent variable. It begins with forward selection with no variables at the hand. The forward selection chooses the variable that contributes most to the discriminatory power as measured by the Wilks' lambda. Each time after a variable is selected, we detect if there are any already selected variable which contributed less discriminatory power as measured by the Wilks's lambda than the significant level proposed. If one such variable is found, it should be removed from the selection. The procedure stops if all the variables selected can not be removed and onne of the other variables can be added. The STEPWISE procedure in SAS was used to conduct the stepwise selection and the results are summarised in the following Table 3.

TABLE 2: Total-sample standardized canonical coefficients on the yellowfin data set generated by SAS. The important feature variables detected by the canonical variate analysisare marked with *.

Chemical elements	γ_1	γ_2	γ_3
NAM*	1.606	0.357	-0.020
SRM*	0.601	-0.140	0.491
KM*	-0.778	-0.064	0.085
SM	-0.023	0.117	-0.289
CLM*	-0.650	0.046	0.139
CAM*	-0.558	-0.122	0.109
FE	-0.040	0.324	0.300
NI	0.168	-0.152	-0.276
RB	-0.004	-0.389	-0.090
ZN*	-0.317	0.806	0.622
CAP*	-0.046	0.466	-0.236
MN	0.016	0.446	0.066
CU	-0.027	0.028	-0.068
BR	0.020	-0.167	0.290
SRP	0.201	-0.076	0.223
SN	-0.098	0.225	0.010
HG	0.232	-0.047	0.429
PB77	-0.087	-0.175	-0.297

TABLE 3: Stepwise feature variable selection on the yellowfin data set using the SAS procedure STEPWISE.

Step	Variable Entered	Removed	F-Statistics	P > F	Wilks's lambda	P < lambda
1	NAM	Nil	26.022	0.0001	0.578	0.0001
2	ZN	Nil	12.822	0.0001	0.424	0.0001
3	KM	Nil	12.278	0.0001	0.314	0.0001
4	SRM	Nil	10.987	0.0001	0.238	0.0001
5	CAM	Nil	5.311	0.0019	0.207	0.0001
6	CLM	Nil	4.424	0.0058	0.183	0.0001
7	CAP	Nil	3.261	0.0246	0.167	0.0001
8	SRP	Nil	2.846	0.0414	0.154	0.0001

Compared the results of Tables 2 and 3, we see that the seven variables with highest canonical coefficients are all selected by the normal distribution based stepwise procedure. The later also select SRP whose canonical coefficient is not very large. The remarkable thing is that both methods confirm that NAM, ZN, KM and SRM are the top four feature variables which lead to the separation of the four groups. Boxplots of the four variables are displayed in Figure 2. It shows that

the average NaM content from the Philippine is well ahead of those of the rest groups. The Philippine group also has lower KM level. This may be a reason why NaM is first selected by the both methods. This corresponds with the fact that the Philippine is well away from rest of the groups as measured by the Mahalanobis distance shown in Table 1. We also see that the Indonesia has much higher Zn content, and the Coral Sea has higher SrM level. The environmental reason behind should be pursued further.

4. Peaking phenomenon

As has been pointed out in the two sections, the main motivatives of variable selection is the so-called "peaking phenomenon". The "peaking phenomenon" says that given a finite size of training data set, the performance of a discrimination rule does not keep on improving as the number p of the feature variables is increased. Rather, after p exceeds certain threshold, the misclassification rate starts to increase. The "peaking phenomenon" is well illustrated analytically by (4.3.2) of McLachlan (1992) based on a training sample of two homogenously normal distributed groups $N(\mu_1, \Sigma)$ and $N(\mu_2, \Sigma)$ with sample size n_1 and n_2 respectively. Let $\Delta = (\mu_1 - \mu_2)^T \Sigma^{-1}(\mu_1 - \mu_2)$ be the Mahalanobis distance between the two groups where the subscript T denots the transpose of a matrix. The formula approximates the error misclassification rate with respect to the group $N(\mu_1, \Sigma)$ as

$$eu(\Delta, n1, n2) \approx \Phi(-\Delta/2) + \{\phi(\Delta/2)/16\}[\{\Delta + 12(p-1)\Delta^{-1}\}n_1^{-1}$$
$$+ \{\Delta - 4(p-1)\Delta^{-1}\}n_2^{-1} + 4(p-1)\Delta/N], \qquad (3.1)$$

where Φ and ϕ are the distribution and density functions of the standard p dimensional normal distribution. The leading term on the right of (3.1), $\Phi(-\Delta/2)$, is determined by the distance Δ between the two groups in a way that the larger the Δ the smaller $\Phi(-\Delta/2)$ is . However, the second term reveals that a larger p value will contribute a increase of the value of the term for fixed n_1 and n_2. These implies that when the increase of dimensionality p after certain threshold can not lead to a significant increase of Δ, the error rate will start to increase.

In the following we shall demonstrate that the current data set exhibits the "peaking phenomenon". We use the cross-validation approach to evaluate the error rate of the Fisher's linear discrimination. Precisely, for each of the 111 data point in the sample we use Fisher's linear discrimination rule constructed from the rest of the data to classify it to see. As the origin of the data point is known, we are able to count how many data point are misclassified and get a error rate. We apply the above cross-validation procedure with 6, 7,8,9 ,10 and all of the variables respectively to observe if there is the "peaking phenomenon". For the cases of 6 - 8 variables, we just use the top 6 - 8 variables from Table 3. We add MN for the case of 9, and further add Fe for the case of 10. The results are summarised in Table 4.

TABLE 4: The "peaking phenomenon" of the data set. Output from the SAS procedure DISCRIM.

No. of variables, p	6	7	8	9	10	18
Error rate	0.286	0.263	0.259*	0.273	0.279	0.357

Table 4 show exactly the "peaking phenomenon". The error rate decreases as the number of feature variables increases. But, after p is larger than 8, the error rate start to increase. This tells us that it is not necessarily good to use all the variables. This is particularly relavent to the fishery study as scientists tend to put lots of variables in their analysis. We should do some pro-selection to see if the discrimination rule performs well with less variables.

5. Data projection

We see from Table 4 of last section that the liner discrimination rule has the best error rate when 8 variables are used. In this section we show that a further lower error rate can be achieved by projecting the data into a three dimension space. The projection is made by transforming each data point x_i of 18 dimensions to $(\gamma_1^T x_i, \gamma_2^T x_i, \gamma_3^T x_i)$ of 3 dimensions , where γ_i $i = 1, 2$ and 3 are the raw canonical coefficients. Please note that the γs here has sightly different meaning from those in Section 3. There the γs were the standardized canonical coefficients. SAS procedure CANDISC is used to get the raw canonical coefficients γs. The projected data set is shown in Figure 3. We see that there are clear clusters of the groups in the projected data. The Philippine is well separated in (1) and (2) of Figure 4. So is the Coral Sea in (3). Even that the Hawaii and the Indonesia are close to each other, they can be separated quite well in (2). Thus we would expect the performance of the linear discrimination analysis should be better. Figure 4 gives boxplots of the projected data set, which shows that it looks very much normal distributed. We employ the SAS procedure DISCRIM on the data and obtain a cross-validation error rate of 19%, which is much lower than the best unprojected error rate of 25.9% as shown in Table 4.

6. Conclusion

We see in this report that the yellowfin tuna from the four birth ground can be well discriminated after careful variable selection and data projection. Without these pro-treatment, the performance of the linear discrimination analysis is at an error rate of 35%, which is too high due to the "peaking phenomenon". By using selecting a subset of the feature variables, the error rate can be largely reduced. It can be further lowered by data canonical projection.

The results shown in this report have important implication on the statistical applications in the fishery research fields, as the scientist there are keen on including as many variables as they can into their data analysis. The signal sent from this report is that using too many variable does not necessary mean good results, as too many variable itself can confuse the real "picture" and generate "noise". Some pro-selection is definitely needed.

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Figure 1. QQ plots of the Mahalanobis distances for the four groups.

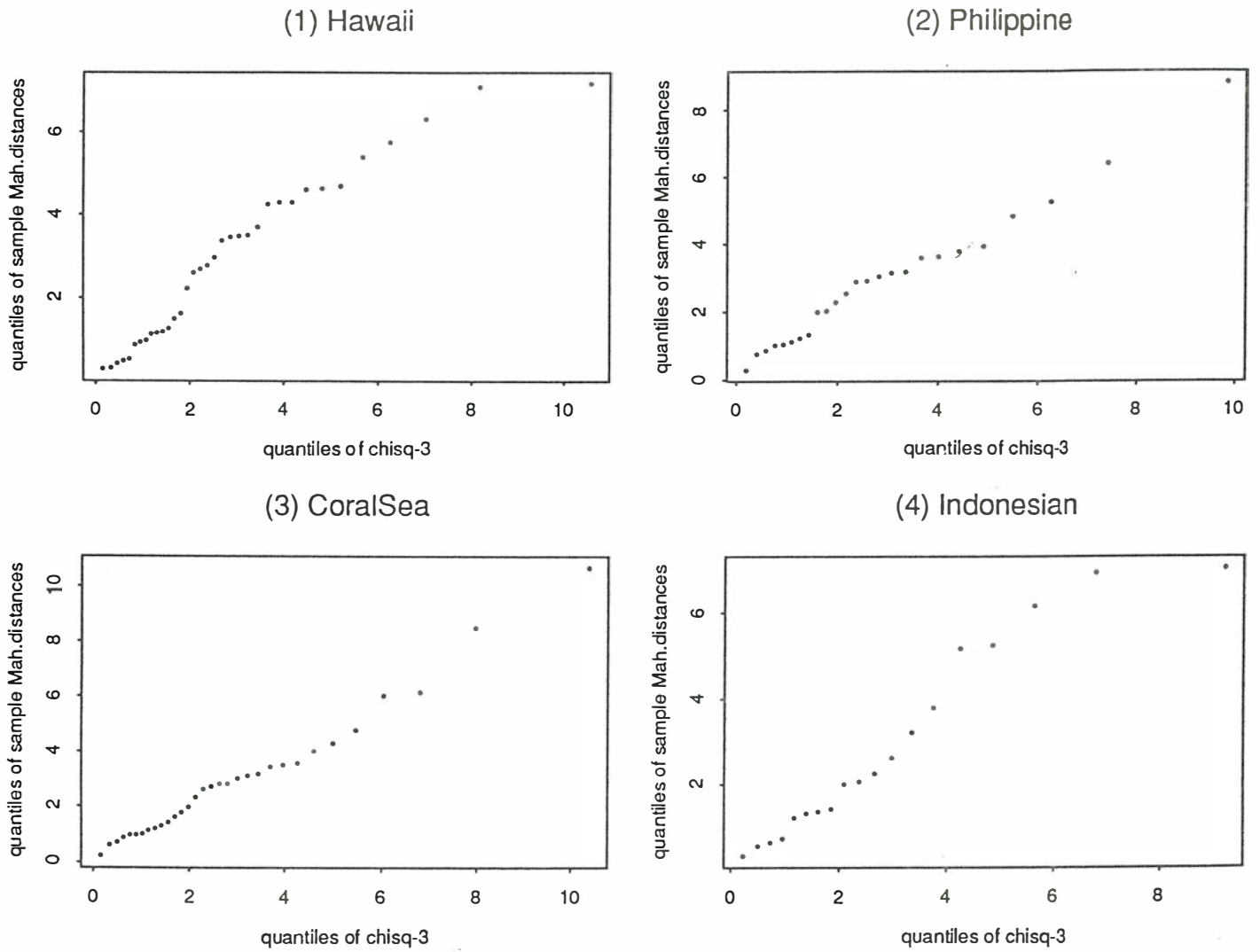(1) Hawaii

(2) Philippine

(3) CoralSea

(4) Indonesian

Figure 2. Boxplots for the four most influential feature variables.
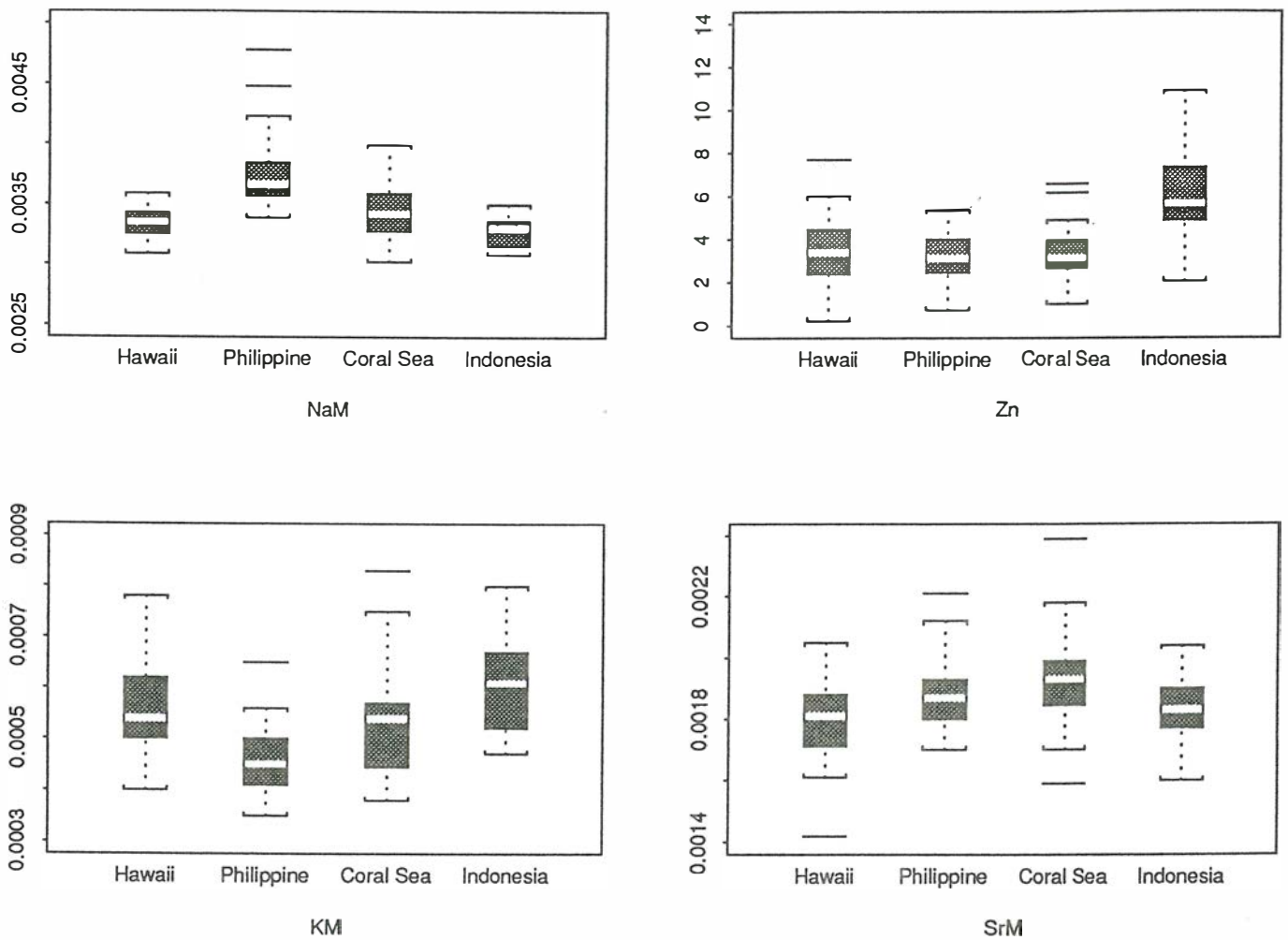
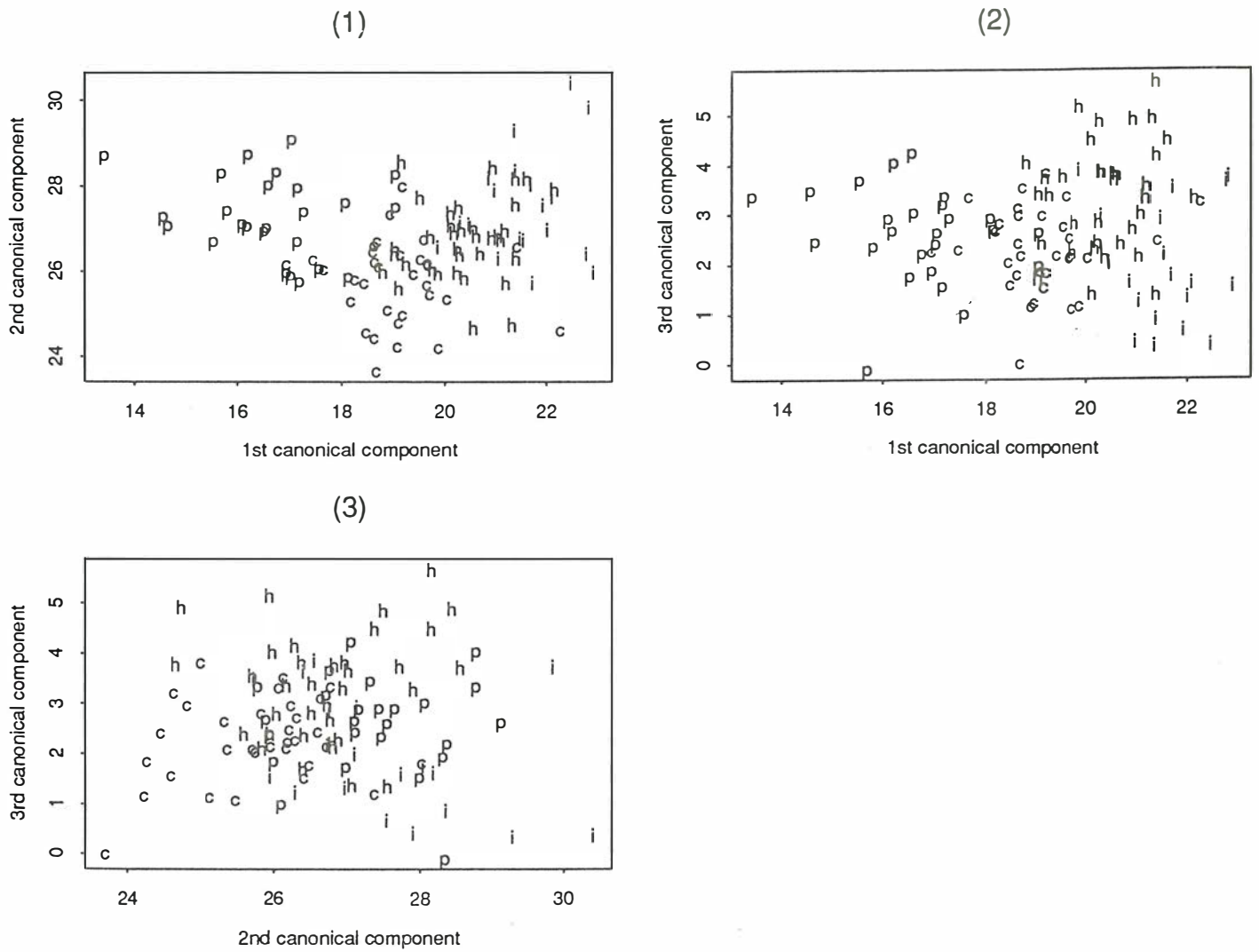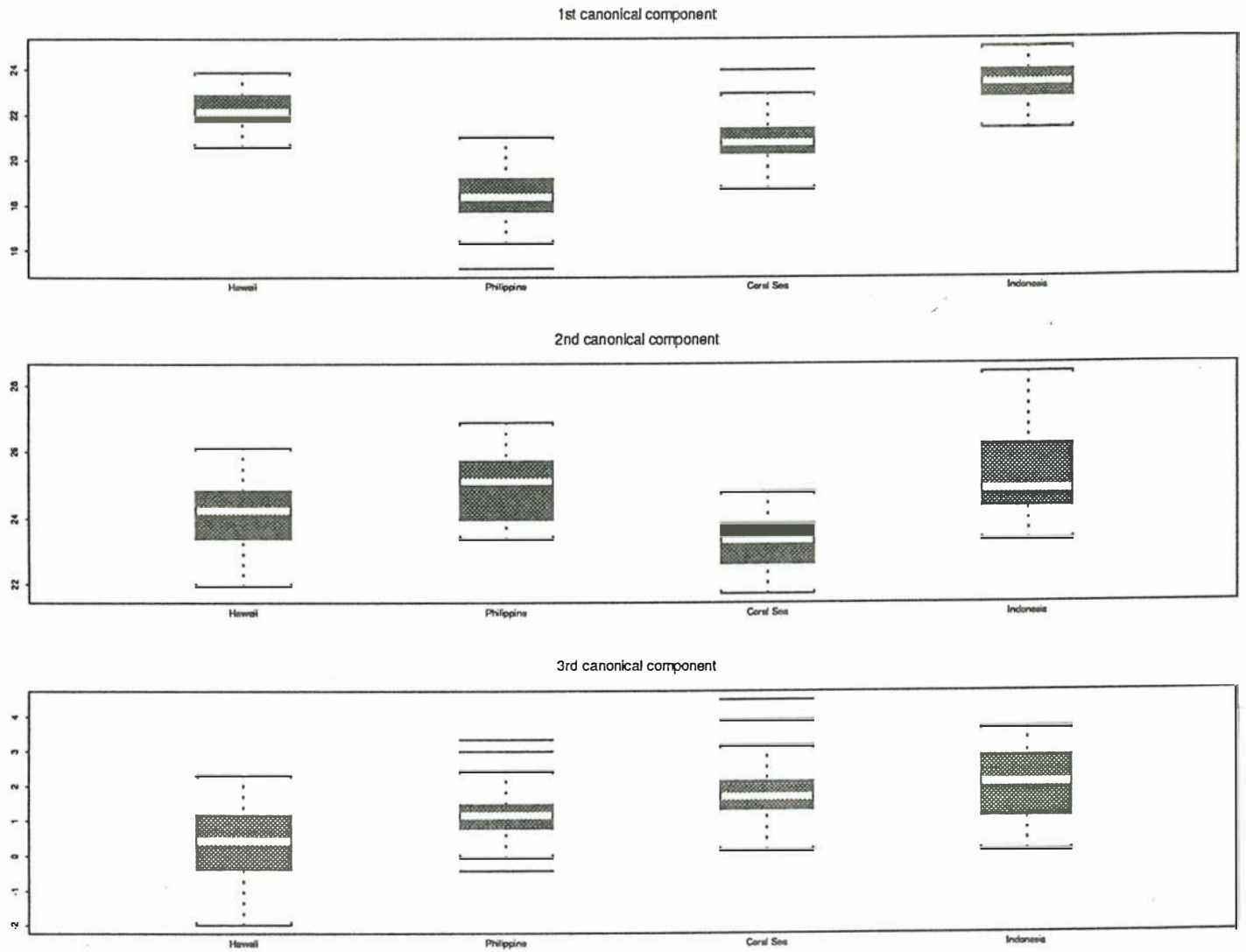Figure 3. Scatter plots of the projected data with group membership.

Figure 4. Boxplots of the projected data

APPENDIX 2. GENETICS *MARINE BIOLOGY* MANUSCRIPT

Allozyme and mitochondrial DNA variation in yellowfin tuna (Thunnus albacares) from the Pacific Ocean

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Running head: Population genetics of yellowfin tuna

Abstract

Samples of yellowfin tuna from the western, central and eastern regions of the Pacific Ocean were examined for genetic variability. Four polymorphic allozyme loci (ADA*, GPI-S*, GPI-F* and FH*) were examined in all samples and a fifth polymorphism (GDA*) examined in western and central samples only. Samples were also screened for mitochondrial DNA variation following restriction analysis by two enzymes (BcI I and Eco RI) detecting polymorphic cut sites. Eighteen mtDNA haplotypes were revealed, with an overall nucleon diversity of 0.678. A subset of animals screened for eight restriction enzymes had an overall nucleon diversity of 0.724 and a mean nucleotide diversity per sample of 0.359%. No significant spatial heterogeneity was detected for alleles at the ADA*, GDA*, GPI-S* and FH* loci nor for the mtDNA haplotypes. Significant heterogeneity was detected for GPI-F*. At this locus, the two eastern samples (southern California and northern Mexico) were not significantly different from each other but were significantly different ($P<0.001$) from the five western/central samples (Coral Sea, Philippines, Kiribati, Hawaii-91 and Hawaii-92). GPI-F*100 was the most common allele in western and central regions, GPI-F*75 the most common in eastern samples.

Introduction

The yellowfin tuna, <u>Thunnus albacares,</u> is a pan-tropical species and the subject of important fisheries in the Indian, Pacific, and Atlantic oceans. In the Pacific, it has a broad distribution, ranging in latitude from 40^ON to 40OS, and is exploited by many nations. In 1991, it is estimated that 259 000 tons were fished from the eastern Pacific Ocean (IATTC, 1992) and in 1992, an estimated 431 000 metric tons were taken from the western, southern and central Pacific region monitored by the South Pacific Commission (Anon., 1993). Clearly this is a major resource, and in Australia at least, it is recognised as one of the few domestic fisheries that might be able to expand in the next decade. Yet knowledge of stock structure of yellowfin tuna remains uncertain, and this is required for the scientifically sound management of the resource.

Suzuki et al (1978) reviewed fisheries and biological data and concluded that there were at least three relatively independent stocks of yellowfin in the Pacific: western, central and eastern. Tagging experiments indicated that most yellowfin move on a scale of hundreds rather than thousands of kilometres (Joseph et al., 1964; Bayliff, 1979; Hunter et al., 1986; Lewis, 1992), and morphometric analysis indicated significant morphometric variation of fish from Japan, Australia, Hawaii, Ecuador and Mexico (Schaeffer, 1991). Despite the fact that spawning appears to be widespread in equatorial regions of the Pacific Ocean, the observed morphometric differentiation and the apparently limited movement of most adult fish suggested that gene flow among subpopulations from widely separated regions of the Pacific may be restricted.

The few population genetic studies carried out on this species have been limited to allozyme analyses. Studies of transferrin variation did not indicate spatial heterogeneity (Barrett and Tsuyuki, 1967; Fujino and Kang, 1968), although a glucose phosphate isomerase polymorphism did reveal significant differentiation of two eastern Pacific samples from one in the western Pacific (Sharp, 1978).

The aim of the present study was to assess levels of genetic differentiation among samples of yellowfin tuna taken from different regions of the Pacific Ocean. This paper

presents the results of the first analysis of mitochondrial DNA variation in this species, and compares these data with the results of an allozyme study carried out on the same samples. It should also be noted that the same individuals (from the western and central Pacific regions only) were examined for variation in otolith microchemistry (Gunn, in prep), and a comparison of the otolith and genetic data is under way.

Materials and Methods.

Sample details are given in Table 1. Tissues were generally taken on board vessels, frozen at $-20^{O}C$ and air freighted frozen to the laboratory where they were stored at $-80^{O}C$. Two of the Hawaii samples (Hawaii-1 and Hawaii-5) came from the Ahui market on Hawaii.

Allozyme analysis.

Allozyme variation was examined using Helena Titan III cellulose acetate plates run with a tris-glycine buffer system (0.02M tris, 0.192M glycine; see Hebert and Beaton 1989 for further details). Small pieces of liver or muscle samples were placed in 1.5ml microcentrifuge tubes, homogenised manually with a few drops of distilled water, and spun in a microcentrifuge for 2 minutes. The supernatant was used for electrophoresis. Following an initial screening, four polymorphic loci were identified from muscle extracts and used in routine analysis. These were ADA* (adenosine deaminase, EC 3.5.4.4), FH* (fumarate hydratase, EC 4.2.1.2) and GPI-F* and GPI-S* (glucose-6-phosphate isomerase, EC 5.3.1.9). Where liver samples were available, GDA* (guanine deaminase, EC 3.5.4.3) was also assayed.

Mitochondrial DNA analysis.

Total DNA was extracted from white muscle tissue using a modified CTAB (hexadecyltrimethylammoniumbromide) protocol described by Grewe et al. (1993).

One hundred and eighteen specimens were screened with 8 restriction enzymes (Bam HI, Ban I, Bcl I, Eco RI, Hind III, Pvu II, Sal I and Xho I). Two of these enzymes (Bcl I and Eco RI), were found to discriminate the majority of haplotypes and were used to screen a total of 434 samples.

The electrophoresis procedure has been described by Grewe et al. (1993). Total DNA was transferred onto a nylon membrane filter (Hybond N+, Amersham Ltd) by southern transfer (Sambrook et al., 1989). The nylon membrane filters were probed with trevalla (Hyperoglyphe antarctica, Teleostei: Stromateoidei) mitochondrial DNA (50 ng used per ten 20cm x 20cm blots) purified by caesium chloride (CsCl) ultracentrifugation. The trevalla probe was labelled with 32P dCTP (Bresatec Pty Ltd) using a GIGAprime DNA labelling kit (Bresatec Pty Ltd). The membrane filters were then exposed to Kodak XAR-5 X-ray film for 12-48 h.

Statistical analysis.

Haplotype (nucleon) and nucleotide diversities within samples, and nucleotide diversities and divergences between samples were computed using the REAP package (McElroy et al., 1992), using the formulations of Nei and Tajima (1981) and Nei (1987).

Allele and haplotype frequency homogeneity across samples was tested using the randomised Monte Carlo chi-square procedure of Roff and Bentzen (1989). This procedure obviates the need to pool rare alleles. For each test, 2000 randomisations of the data were carried out, each giving a randomised chi-square value (χ^2_{null}). The probability that the null hypothesis of genetic homogeneity was correct was given by $P = n/2000$, where n was the number of randomisations that generate $\chi^2_{null} \geq \chi^2$ and where χ^2 is the chi-square value given by the actual observations. .

The extent of genetic differentiation among samples was quantified using Nei's gene diversity statistic G_{ST} (Nei, 1973), which reflects the proportion of total genetic variation attributable to differentiation between populations. For each locus it was estimated as $(H_T - H_S)/H_T$, where H_T represents total heterozygosity and H_S is

average (Hardy-Weinberg expected) population heterozygosity. The proportion or magnitude of G_{ST} generated by sampling error, which we have termed $G_{ST.null}$, was estimated using a bootstrapping program, given the observed allele or haplotype frequencies and sample sizes (Elliott and Ward, 1992). Simulations were run 2000 times to provide a mean value of $G_{ST.null}$ and a standard deviation. The probability of obtaining a value of $G_{ST.null}$ as large or larger than that obtained from the actual observations, G_{ST}, was given by $P=n/2000$, where n is the number of randomisations that generate $G_{ST.null} \geq G_{ST}$. Values of P less than 0.05 indicated significant differentiation between areas that could not be explained by sampling error alone.

Probability corrections for multiple tests were carried out using both the (conservative) standard Bonferroni procedure (Miller, 1980; Lessios, 1992) and the more powerful sequential Bonferroni procedure of Hochberg (1988; see also Lessios, 1992). The predetermined significance level, α (where $\alpha = 0.05$), was adjusted using each of these methods to obtain corrected significance levels, α_B' and α_H' respectively.

Results

The five small samples taken from the region of Kiribati (Kiribati, Nikunau, Baker Isle, Nikumaroro and Marshall Islands, see Table 1) did not show significant differentiation at any of the genetic markers examined and were pooled in all ensuing analyses. The four small 1991 Hawaii samples similarly showed no differentiation and were subsequently pooled as Hawaii-91. The two Hawaii 1992 samples were pooled as Hawaii-92. After pooling, seven samples remained for statistical analysis.

Allozymes

Allele frequencies and sample sizes of the seven samples are given in Table 2. Genotype numbers at all loci in all seven samples accorded with Hardy-Weinberg expectations ($P>0.05$). Samples were tested using χ^2 analysis with single degrees of

freedom, having first pooled rare alleles in order to reduce the number of genotype classes with small expectations.

The four polymorphic allozyme loci examined in each of the seven samples and the one locus examined in three samples were then tested for allele frequency heterogeneity (Table 3) using the Roff and Bentzen (1989) procedure. ADA* and FH* frequencies showed no significant heterogeneity ($P>0.05$). GPI-S* showed heterogeneity at $P=0.040$, but for this locus $\alpha_B' = 0.010$ and $\alpha_H' = 0.017$: it was therefore concluded that there was no significant heterogeneity. GDA* was only screened in three samples, and showed heterogeneity at $P=0.011$. This compares with $\alpha_B' = 0.010$ and $\alpha_H' = 0.013$. Thus the allele frequency distribution at this locus is marginally significant, but this marginal significance is largely due to the presence of two copies of the GDA*80 allele in the Hawaii-91 sample and its absence from the Phillipines and the Coral Sea samples. Ignoring this allele results in a non-significant probability (0.091). GPI-F* showed clear evidence of spatial heterogeneity at $P<0.001$, a probability much lower than α_B' (0.010) and α_H' (0.013).

The source of this heterogeneity was identified by carrying out all possible pairwise population comparisons of allele frequencies (Table 4), again using the Roff and Bentzen (1989) procedure. A total of 21 comparisons were performed, yielding an α_B' value of 0.0024. The California and Mexico samples were not significantly different from each other ($P=0.289$), but both were significantly different from each of the other samples (one comparison of $P=0.001$, 9 comparisons where $P<0.001$). This conclusion also held when the sequential Bonferroni adjustment procedure was adopted. There was a suggestion that the Hawaii-92 sample differed slightly from the Hawaii-91 sample ($P=0.013$), and that the Hawaii-92 sample also differed from the Coral Sea sample ($P=0.018$), although neither of these comparisons was significant following Bonferroni adjustment.

The extent of genetic population differentiation in our data was quantified by the use of G_{ST} statistics (Nei, 1973). For four of the loci (ADA*, FH*, GPI-S* and GDA*), about 1% of the allele frequency differentiation could be attributed to sample

differentiation, but boot strapping showed that given the sample sizes this could have arisen from sampling error alone (Table 4). Note that the marginally significant heterogeneity of the locus GDA* revealed in the chi square analysis was primarily attributable to rare allele differentiation; such differentiation will have a very small effect when apportioning total variation into among and within sample components in a G_{ST} analysis and this accounts for the non-significant result derived from the latter analysis. The fifth locus, GPI-F*, shows that about 10% of its differentiation can be attributed to an among sample component, a value much larger than the estimate of around 1% attributable to sampling error alone ($P<0.001$). This result accords with the conclusions of the heterogeneity chi-square analysis.

Sharp (1978) also studied variation at the GPI-F* locus, and his data are compared with ours in Table 5. His western sample, from the Bismarck Sea (off the northern coast of Papua New Guinea), had allele frequencies that were not significantly different from our pooled western/central Pacific sample ($P>0.05$). Similarly, there was no significant heterogeneity between his samples off Mexico (Roca Partida) and off Ecuador, and our pooled California/Mexico sample ($P>0.05$). In the western/central Pacific samples, GPI-F*100 had an allele frequency in excess of 0.5 and GPI-F*75 a frequency less than 0.4, whereas in the eastern Pacific samples these frequencies are reversed, GPI-F*100 having a frequency less than 0.4 and GPI-F*75 a frequency greater than 0.5. In addition, GPI-F*135 has a frequency of less than 0.05 in the central/western region but around 0.10 in the east.The GPI-F* allele frequencies in the 10 samples (7 from our study, three from Sharp 1978) are depicted in Figure 1. A clear allele frequency discontinuity is observed between the western/central samples and those from the eastern Pacific. However, additional data are required from the region of the Marquesas Islands and/or Tahiti to determine whether there is an abrupt frequency change or a steep cline in this region.

We pooled our data with those of Sharp (Table 5), and for the pooled western/central versus pooled eastern samples estimated a G_{ST} value of 0.079 (with a $G_{ST.null}$ estimate 0.001± 0.001, $P<0.001$). This equates to an $N_{e}m$ estimate of around 3 individuals per generation, using the island model of migration (Wright, 1943):

$$F_{ST} = 1/(1 + 4N_em) \quad \text{or} \quad N_em = 0.25 \, (1/F_{ST} - 1),$$

where $F_{ST} = G_{ST}$. This relationship between F_{ST} or G_{ST} and N_em is approximately true if $m \ll 1$, $\mu \ll m$, and if the populations are at equilibrium (N_e is the effective population size, μ is the mutation rate and m is the rate of gene flow per generation). It assumes selective neutrality of the different genotypes, permitting population differentiation to be attributed solely to genetic drift and migration.

Mitochondrial DNA

The two restriction enzymes examined in 434 fish revealed a total of 18 haplotypes (Table 2). Two of these, AA and AB, accounted for about 80% of all haplotypes (34.1% and 43.5% respectively). Only two other haplotypes were present at frequencies greater than 5% (BB, 5.7%; CB, 6.9%). Nucleon diversities ranged from 0.540 to 0.712 per sample (overall 0.678). These values were only a little less than those given by the eight enzyme survey (overall 0.724), showing that these two enzymes revealed the bulk of the variation detected by the eight enzymes. Neither the chi-square analysis nor the G_{ST} analysis of the two enzyme survey revealed significant sample differentiation (Table 3, $P > 0.05$). There is no evidence of particular haplotypes being restricted to particular locations, although 16 of the 18 observed haplotypes were rare and greatly increased sample sizes would be necessary to rule out this possibility. Since the GPI-F* analysis had revealed the existence of two genetically distinguishable groups, western/central and eastern Pacific, the mitochondrial DNA data were analysed in a similar fashion. There was no significant differentiation in haplotype frequencies between these two pooled samples ($P > 0.05$).

Haplotypes, haplotype frequencies, and nucleon and nucleotide diversities of the subset of samples examined using eight restriction enzymes are given in Table 6. A total of 15 haplotypes were observed. Nucleon diversities ranged from 0.627 to 0.809 (overall 0.724). Nucleotide diversities ranged from 0.258% to 0.427% (mean 0.359%),

with nucleotide divergences between pairs of samples (corrected for nucleotide diversities within samples) ranging from 0.006% to -0.008% (mean -0.004%). Neither the chi square analysis nor G_{ST} analysis (treating haplotypes as alleles) revealed significant inter-sample differentiation (P>0.05).

Fragment sizes for the haplotypes listed in Tables 2 and 6 are given in Table 7. The mean mtDNA size is estimated as 16 856 base pairs (SD=396).

Discussion

Mitochondrial DNA analysis is generally considered a more powerful tool than allozyme analysis for revealing genetic population structure. This is because the effective population size of mitochondrial DNA, being a haploid and maternally transmitted molecule, is only one quarter that of nuclear genes and is thus more susceptible to genetic drift (Nei and Lei 1979; Birky et al., 1989). In addition, mitochondrial DNA is generally held to evolve an order of magnitude faster than single copy nuclear DNA (Brown et al., 1979), although it has been suggested that poikilotherms show a reduced rate of mitochondrial DNA evolution (Martin et al., 1992). Most studies in the past have revealed greater mitochondrial DNA than allozyme differentiation among populations (eg Avise et al., 1979; Lansman et al., 1983; Saunders et al., 1986; Ward et al., 1989, Reeb and Avise, 1990).

This study has shown the reverse. Significant genetic heterogeneity was detected among yellowfin tuna samples taken from different regions of the Pacific Ocean, but this was confined to one (GPI-F*) of the five polymorphic allozymes assayed, with no significant heterogeneity among the 18 mitochondrial haplotypes. Nuclear DNA differentiation can exceed mitochondrial DNA differentiation when either the migration rate or the breeding sex ratio is strongly biased towards females (Birky et al., 1989). Neither of these conditions appears to hold for yellowfin tuna, and in fact there is a preponderance of males among fish larger than 130 cm (IATTC, 1991). There is thus no reason to expect that nuclear DNA differentiation of yellowfin tuna should exceed that of

mitochondrial DNA, and it seems probable that further mtDNA analysis would reveal significant population differentiation. Alternatively, it could be argued that there is extensive gene flow across the Pacific, and that the GPI-F* differentiation results from local selective processes. Such an interpretation is not, however, supported by extensive tagging data (see later). In any case, this result re-affirms the wisdom of carrying out, whenever possible, both mitochondrial and allozyme surveys in studies of stock delineation.

The bulk of the observed GPI-F* differentiation separated eastern Pacific (California and Mexico) samples of yellowfin tuna from samples taken from the central and western Pacific. The identification of the Californian / Mexican fish as a single genetic stock (or, more correctly stated, the failure to reject the hypothesis that they are different stocks) is consistent with the presence of a relatively discrete and continuous yellowfin spawning area (as determined from the existence of larval fish) along the eastern Pacific coastline, extending from about $25^{O}N$ to the equator (Suzuki et al, 1978). Sharp's (1978) data from a more southerly Mexican sample and one from Ecuador also indicate no genetic differentiation in this region, and allele frequencies of his samples were not distinguishable from our Mexican and Californian samples. Schaefer (1991) showed that while samples of adult yellowfin from Mexico and Ecuador were morphologically distinguishable, there was more overlap between these two localities than between samples from the Mexico/Ecuador region and from Australia, Japan and Hawaii. He suggested that there may be greater mixing of fish within the eastern Pacific than with fish from the central/western regions. This suggestion is consistent with the results of tagging studies, which show that movements of yellowfin tuna in the eastern Pacific are generally restricted to the region of a few hundred miles, with no long distance movements from the eastern to the western or central Pacific (Joseph et al., 1964; Bayliff, 1979; Hunter et al., 1986). The genetic, larval, and tagging data all indicate that fish in the eastern Pacific are likely to share a single gene pool, and it is probable that the morphological differences between yellowfin from Ecuador and Mexico (Schaefer 1991) reflect environmental rather than genetic variation.

There was no clear genetic separation of fish from central and western Pacific

regions, including the sample of Sharp (1978) from the Bismarck Sea. In the central/western region, there was a suggestion that the Hawaii-92 fish were slightly different from the Hawaii-91 and Coral Sea samples, but this conclusion does not stand up to Bonferroni adjustments of probability levels. The sample from the Philippines comprised fish in the region of three to nine months of age, while those from Kiribati were about one year old. These fish were possibly still in the vicinity of their natal spawning grounds and yet showed no significant differentiation, despite being separated by about 6,500 km. There appears to be sufficient gene flow among populations in the central/western Pacific to maintain the genetic integrity of the gene pool in this vast area, but sampling of additional localities and increased sample sizes are required to further test this conclusion.

Meristic and morphological examination of samples in the western/central Pacific region from New South Wales (Australia), Ishigaki (Japan) and Oahu (Hawaii) revealed significant differences, suggesting that fish from these areas formed distinct groups (Schaeffer, 1991). However, there was some overlap in the characteristics of Australian and Japanese fish, and of Japanese and Hawaiian fish, and these data do not rule out the possibility of small numbers of individuals moving between these areas. Indeed, while most movements appear to be on a relatively local longitudinal scale, there are also numerous records of yellowfin moving from sub-tropical to Japanese (temperate) waters (Lewis, 1992). Two major areas of high larval densities in the western/central equatorial Pacific have been described ($130^{O}E$-$170^{O}E$ and$130^{O}W$-$160^{O}W$), but spawning appears to be generally widespread (Suzuki et al., 1978). In this part of the Pacific, movements of a few hundred kilometres of most fish, taken together with the more extensive movements of a minority of fish and the presence of widespread spawning, probably provides sufficient gene flow to account for the lack of genetic heterogeneity.

The results of this study indicate clearly that there are at least two genetically different groups of yellowfin tuna in the Pacific Ocean, one comprising eastern Pacific fish (California, Mexico, Ecuador) and the other western/central Pacific fish (Coral Sea, Kiribati, Philippines, Papua New Guinea, Hawaii). These groups can be differentiated

on the basis of GPI-F* genotype. Furthermore, the significant spatial genetic heterogeneity has been temporally stable, at least over the last 15 years. It seems likely that gene flow between these two groups is severely restricted, with perhaps just a few individuals per generation migrating between these two regions. Assuming that the observed differentiation at GPI-F* is the result of genetic drift, then the lack of differentiation of the four other polymorphic loci (although one of these, GDA*, was not sampled in eastern populations) and the mitochondrial DNA variants suggests that this separation is, in evolutionary terms, relatively recent.

Although the genetic data indicate the existence of two reproductively isolated groups in the Pacific, catch data do not indicate any break in the distribution of yellowfin tuna across the equatorial region (Suzuki et al. 1978, Lawson, 1992). However, yellowfin larvae are found in higher density nearer islands (Leis et al., 1990, Lawson, 1992), and the relative absence of islands in the eastern Pacific and their abundance in the western/central region could account for the genetic isolation of fish of these two areas. The spatial heterogeneity observed on a broad scale offers some hope that with an increased sampling effort more fine-scale heterogeneity may be demonstrable. It would also be worthwhile to extend the sampling to yellowfin in the Atlantic and Indian Oceans to get a more global perspective of population structure. Despite the lack of success of mitochondrial DNA analysis in revealing population differentiation, such approaches should be persevered with and renewed attempts made to find additional restriction enzymes detecting polymorphic sites. The use of both allozyme and mitochondrial DNA analysis will inevitably give deeper insights into population structure than the use of either technique alone.

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Table 1. <u>Thunnus albacares.</u> Sample details

Location	Approximate lat./long.	Date	n	size[a]	tissue[b]
Philippines	8°N 119°E	March 1991	47	32-46 cm	m,l
Coral Sea	15°N 146°E	October/November 1991	100	49-111 cm	m,l
Kiribati	0°N 173°E	August 1991	29	52-67 cm	m
Nikunau	2°N 179°E	March 1992	35	not available	m
SW Baker Isle	2°N 177°E	April 1992	9	not available	m
NW Nikumaroro	2°N 176°E	April 1992	15	50-55 cm	m
Marshall Islands	5N 173E	August 1991	6	51-88 cm	m
Hawaii-1	20°N 155°W	August 1991	17	120-180cm	o,t
Hawaii-2	20°N 155°W	October 1991	19	620-840 g	m,l
Hawaii-3	20°N 155°W	November 1991	4	330-590 g	m,l
Hawaii-4	20°N 155°W	December 1991	14	280-1020 g	m,l
Hawaii-5	20°N 155°W	August 1992	80	c. 60-180 cm	m
Hawaii-6	20°N 155°W	August 1992	6	c. 60-180 cm	m
California	33°N 117°W	August 1992	41	c. 50-70 cm	m
Mexico	29°N 117°W	June 1992	40	not available	m

[a] lengths given in preference to weights, when available

[b] m=white muscle, l=liver, o=ovary, t=testis

Table 2. <u>Thunnus albacares.</u> Allele and mitochondrial DNA haplotype frequencies (<u>Bcl</u> I and <u>Eco</u> RI haplotypes respectively), and sample sizes. Nucleon diversity (<u>h</u>) is also given for the mtDNA data.

Locus	allele/ haplotype	Philippines	Coral Sea	Kiribati	Hawaii-91	Hawaii-92	California	Mexico
ADA*	115	0.298	0.306	0.399	0.338	0.372	0.317	0.359
	100	0.638	0.638	0.567	0.635	0.596	0.671	0.628
	85	0.064	0.056	0.034	0.027	0.032	0.012	0.013
	n	47	98	89	37	78	41	39
FH*	130	0.096	0.117	0.086	0.014	0.104	0.051	0.075
	100	0.893	0.878	0.900	0.972	0.896	0.949	0.925
	75	0.011	0.005	0.014	0.014	-	-	-
	n	47	98	70	35	77	39	20
GPI-S*	-20	-	-	-	0.014	-	-	-
	-60	0.213	0.163	0.233	0.135	0.103	0.187	0.231
	-100	0.787	0.837	0.767	0.851	0.891	0.813	0.769
	-125	-	-	-	-	0.006	-	-
	n	47	98	88	37	78	40	39
*GPI-F**	135	0.033	0.036	0.011	0.014	0.032	0.122	0.077
	100	0.565	0.683	0.673	0.743	0.545	0.305	0.231
	75	0.391	0.281	0.316	0.243	0.423	0.573	0.692
	40	0.011	-	-	-	-	-	-
	n	46	98	87	37	78	41	39
GDA*	120	0.170	0.108	-	0.100	-	-	-
	110	0.330	0.366	-	0.243	-	-	-
	100	0.479	0.500	-	0.543	-	-	-
	90	0.021	0.026	-	0.085	-	-	-
	80	-	-	-	0.029	-	-	-
	n	47	97	-	35	-	-	-
mtDNA	AA	0.412	0.340	0.443	0.314	0.254	0.294	0.325
	AB	0.412	0.402	0.364	0.608	0.494	0.463	0.425
	AC	-	-	0.011	0.027	0.012	-	-
	AF	-	-	-	-	0.012	0.049	0.025

Table 2, continued.

AG	-	-	-	-	0.012	-	-
BA	0.029	0.010	-	0.020	-	0.049	-
BB	0.059	0.052	0.068	0.020	0.084	0.073	0.025
CA	0.029	0.062	0.011	-	0.024	0.024	0.050
CB	0.059	0.103	0.057	0.020	0.060	0.024	0.125
DB	-	0.010	-	-	-	-	-
EB	-	0.021	-	-	-	-	-
LB	-	-	0.011	-	-	-	-
MB	-	-	0.011	-	-	-	-
NB	-	-	-	-	0.012	-	-
PB	-	-	-	-	0.024	0.024	-
OA	-	-	-	-	-	-	0.025
OB	-	-	0.023	-	-	-	-
QB	-	-	-	-	0.012	-	-
n	34	97	88	51	83	41	40
h	0.672	0.712	0.670	0.540	0.688	0.705	0.712

Table 3. <u>Thunnus albacares.</u> Analyses of genetic differentiation among samples. Note that the mtDNA analysis is for the two-enzyme examination.

Locus	samples	number of alleles/ haplotypes	heterogeneity χ^2 analysis χ^2	P	G_{ST}	genetic diversity analysis $G_{ST.null} \pm SD$	P
ADA*	7	3	11.649	0.488	0.005	0.008±0.004	0.753
FH*	7	3	12.784	0.375	0.013	0.010±0.006	0.240
GPI-S*	7	4	27.983	0.040	0.013	0.008±0.005	0.150
GPI-F*	7	4	106.033	<0.001	0.106	0.008±0.005	<0.001
GDA*	3	5	19.145	0.011	0.008	0.007±0.005	0.248
mtDNA	7	18	106.412	0.350	0.019	0.016±0.006	0.257

Table 4. <u>Thunnus albacares.</u> Pairwise sample comparisons of allele frequencies at the <u>GPI-F</u>* locus. Probabilities of no heterogeneity above the diagonal, chi square values below.

	Philippines	Coral Sea	Kiribati	Hawaii-91	Hawaii-92	California	Mexico
Phillipines	-	0.099	0.101	0.079	0.644	0.001	<0.001
Coral Sea	5.936	-	0.287	0.496	0.018	<0.001	<0.001
Kiribati	5.402	2.630	-	0.640	0.040	<0.001	<0.001
Hawaii-91	6.205	1.443	1.329	-	0.013	<0.001	<0.001
Hawaii-92	1.882	7.853	6.392	8.350	-	<0.001	<0.001
California	15.170	35.021	37.324	31.224	16.141	-	0.289
Mexico	21.104	46.401	44.495	40.247	21.233	2.526	-

Table 5. <u>Thunnus albacares.</u> Comparison of <u>GPI-F</u>* allele frequencies in ours and Sharp's (1978) data sets.

Locus	allele[a]	western / central Pacific			eastern Pacific			
		our data[b]	Bismarck Sea[c]	**pooled data**	our data[d]	Roca Partida[c]	Ecuador[c]	**pooled data**
GPI-F*	135 (1)	0.026	0.005	**0.018**	0.100	0.097	0.127	**0.109**
	100 (2)	0.640	0.667	**0.650**	0.269	0.327	0.335	**0.318**
	75 (3)	0.332	0.326	**0.330**	0.631	0.573	0.538	**0.571**
	40 (4)	0.002	0.002	**0.002**	-	0.003	-	**0.001**
	n	346	213	559	178	155	80	413

[a] Sharp (1978) terminology given in parentheses
[b] pooled samples from the Coral Sea, Philippines, Marshall Islands, Kiribati, Hawaii.
[c] data from Sharp, 1978.
[d] pooled northern Mexico and southern California samples.

Table 6. <u>Thunnus albacares.</u> Haplotype frequencies and nucleon (<u>h</u>) and percent nucleotide (<u>nd</u>) diversities of the four populations examined using eight restriction enzymes. Haplotype designations reflect restriction profiles for the following enzymes: <u>Bam</u> HI, <u>Ban</u> I, <u>Bcl</u> I, <u>Eco</u> RI, <u>Hind</u> III, <u>Pvu</u> II, <u>Sal</u> I and <u>Xho</u>, respectively.

	Philippines	Coral Sea	Kiribati	Hawaii-91
AAAAAAAA	0.304	0.346	0.345	0.400
AAAAABAA	-	-	-	0.025
AAABAAAA	0.391	0.231	0.379	0.475
AAABBAAA	-	-	0.034	-
AAACAAAA	-	-	0.034	0.025
AABAAAAA	0.043	0.038	-	0.025
AABBAAAA	0.087	0.038	0.034	0.025
AACAAAAA	0.043	0.038	-	-
AACBAAAA	0.043	0.192	0.069	0.025
AALBAAAA	-	-	0.034	-
AAMBAAAA	-	-	0.034	-
ABABAAAA	0.043	-	-	-
ADAAAAAA	-	0.038	0.034	-
BAAAAAAA	0.043	-	-	-
BAABAAAA	-	0.077	-	-
<u>n</u>	<u>23</u>	<u>26</u>	<u>29</u>	<u>40</u>
<u>h</u>	<u>0.771</u>	<u>0.809</u>	<u>0.751</u>	<u>0.627</u>
<u>nd(%)</u>	<u>0.375</u>	<u>0.427</u>	<u>0.375</u>	<u>0.258</u>

Table 7. Thunnus albacares. Fragment sizes for each restriction profile produced by the polymorphic enzymes used in the present survey. Sal I did not cleave the yellowfin mtDNA and is not included in this table. -: absence of fragment

Bam HI

A	B
-	16,800
13,850	-
2,950	-
16,800	16,800

Ban I

A	B	D
5,237	5,237	-
-	4,881	-
-	-	4,350
3,495	3,495	3,495
3,416	3,416	3,416
2,767	-	2,767
2,114	-	2,114
-	-	887
17,029	17,029	17,029

Bcl I

A	B	C	D	E	L	M	N	O	P	Q
-	-	-	-	-	9,682	-	-	-	-	-
-	-	-	-	-	-	-	8,656	-	-	-
-	7,818	-	7,818	7,818	-	-	-	-	-	-
4,796	-	4,796	-	-	-	4,796	4,796	4,796	4,796	4,796
4,560	4,560	-	-	-	-	-	-	-	-	-
4,096	4,096	4,096	4,096	4,096	4,096	4,096	-	4,096	4,096	4,096
-	-	-	-	-	-	-	-	3,890	-	-
-	-	-	-	3,600	-	-	-	3,600	3,600	-
-	-	-	3,120	-	-	-	-	-	-	-
3,022	-	3,022a	-	-	3,022	3,022	3,022	-	3,022	3,022
-	-	-	-	-	-	-	-	-	-	2,990
-	-	-	-	-	-	1,672	-	-	-	-
-	-	1,580	-	-	-	1,580	-	-	1,580	1,580
-	-	-	-	-	-	1,350	-	-	-	-
-	-	-	1,100	-	-	-	-	-	-	-
-	-	-	-	650	-	-	-	650	-	-
564	564	564	564	564	-	564	564	564	-	564
17,038	17,038	17,080	16,698	16,728	16,800	17,080	17,038	17,596	17,094	17,048

Table 7, continued

Eco RI

A	B	C	F[b]	G[b]
9,070	9,070	9,070	-	-
7,750	-	-	-	-
-	-	-	-	7,038
-	-	-	4,550	-
-	-	-	4,350	-
-	-	4,100	-	-
-	3,890	-	3,890	3,890
-	3,520	-	3,520	3,520
-	-	3,150	-	-
16,820	16,480	16,320	16,310	16,480

Hind III

A	B
4,834	4,834
3,669	3,669
2,997	2,997
-	3,210
1,686	-
1,477	-
1,395	1,395
16,058	16,105

Pvu II

A	B
9,070	9,070
6,790	-
-	4,290
-	2,500
1,650	1,650
17,510	17,510

Xho I

A
11,900
4,850
16,750

[a] Presumed doublet

[b]Seen in fish not examined for all eight enzymes and therefore not listed in Table 6.

Figure 1. <u>Thunnus albacares.</u> Allele frequencies at the <u>GPI-F</u>* locus. The larger circles represent our data, the smaller circles those of Sharp (1978).

Hawaii 91

California

Philippines

Bismark Sea

Hawaii 92

Mexico

Kirribati

Roca Partido

Coral Sea

Argentina

75   135

100

*GPI-F** alleles
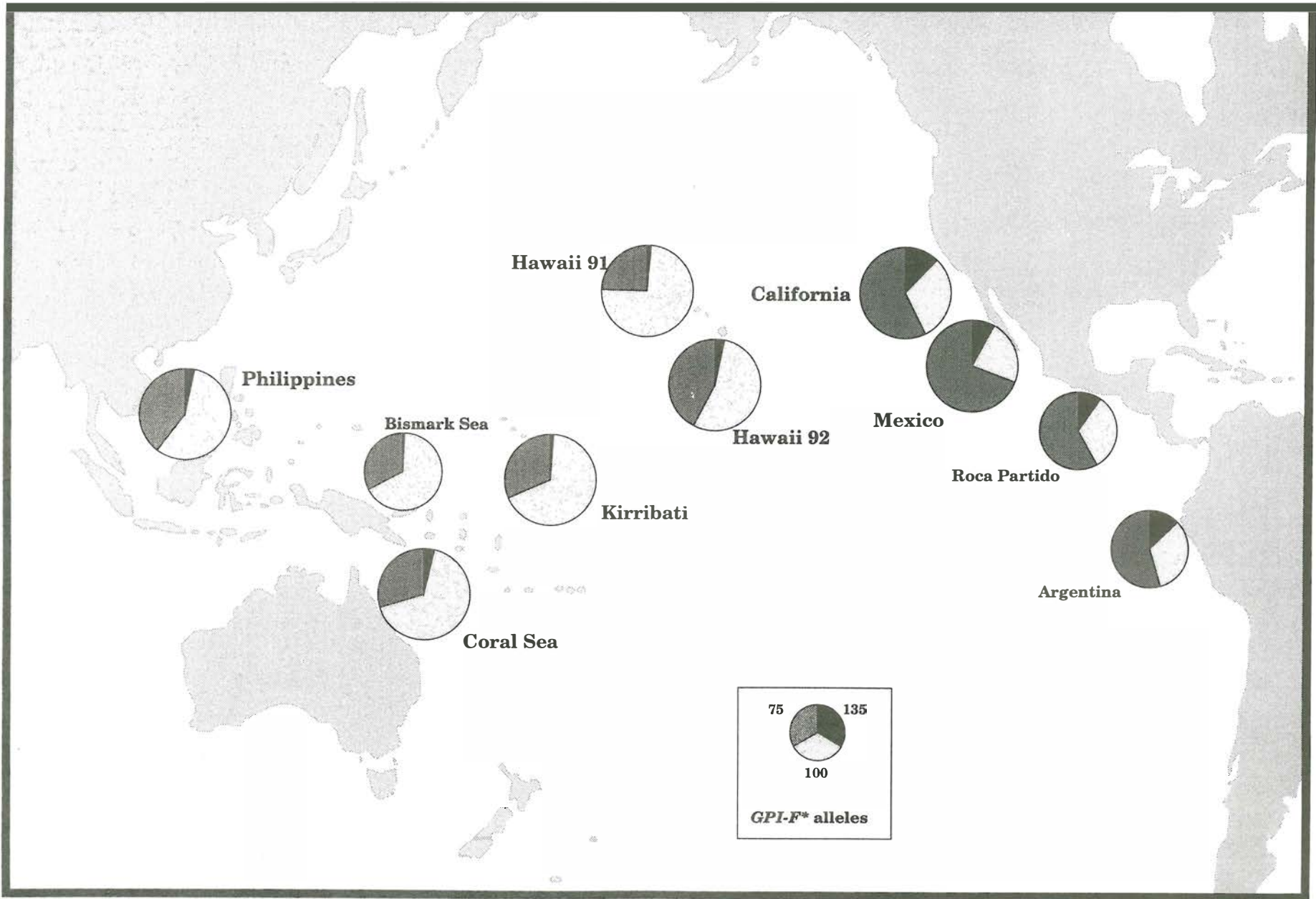
APPENDIX 3: FIRDC PROPOSAL

SECTION 1 – PROJECT TITLE

The discrimination of yellowfin tuna sub-populations exploited within the AFZ. Phase I - A pilot study to determine the extent of genetic and otolith microchemical variability in populations from different parts of the Pacific and Indian Oceans.

SECTION 2 – KEYWORDS

Yellowfin tuna, identification, genetics, otolith microchemistry.

SECTION 3 – OBJECTIVES

As the proposed project is intended as a pilot study to a subsequent larger research project dealing with the links between yellowfin spawning grounds in the Coral Sea and the recruitment to domestic and foreign commercial fisheries off the east coast of Australia, it has limited but well defined objectives. These are as follows:

1. To provide an allozyme-based taxonomic key to the early life history stages of *Thunnus* species occurring in eastern Australian waters. This key would provide, for the first time, an accurate and efficient means of separating larval, post-larval and small juvenile specimens of the principal commercial tuna species (e.g. yellowfin, bigeye, albacore, southern bluefin, skipjack).

2. To assess the genetic variability in larval/juvenile yellowfin tuna collected from four different locations; one off the north-east coast of Australia, two in other parts of the Western Pacific and one in the Indian Ocean,

3. Using the specimens for which genetic data have been collected, to assess the extent of variability in otolith microchemistry, particularly in that region of the otolith that is deposited during the early stage of larval development (i.e. while the larvae are close to the spawning area). Data on the variability among and within samples from the four areas will provide a means of assessing whether yellowfin tuna otoliths constitute a natural tag that will allow the identification of spawning areas from which recruits to domestic and foreign fisheries within the AFZ originate.

SECTION 4 – JUSTIFICATION

The yellowfin tuna fishery off Australia's east coast has developed rapidly during the 1980s and in 1988-89 involved 45-170 boats catching nearly 1000 tonnes, worth in the order of $5 million (pers. comm. BRR 1990). Yellowfin is also the target of recreational fishing, especially off NSW where about 10,000 anglers are involved. It is recognized as one of the few domestic fisheries with the potential to expand in the next decade. In addition to the domestic catch, 3700 tonnes of yellowfin tuna, worth about $15 million, were caught in 1988-89 by Japanese longliners operating within the AFZ (pers. comm. BRR 1990). However, the combined Japanese-Australian catch within the AFZ is small when compared with the 650,000 tonnes of yellowfin taken by Japanese, Taiwanese, Korean and US fishermen throughout the Central and Western Pacific. Clearly, within the Western Pacific region, which includes the tropical waters of the Coral Sea within the AFZ, there is a very large yellowfin population.

With the considerable increases in effort in the Australian fishery over the past 5 years and the likelihood of further expansion and capitalization, particularly in the off-shore sectors of the zone, the definition of population sub-structure of yellowfin within the AFZ is a critical prerequisite for the development of management policy. Whether or not the current domestic fishery exploits a separate, "Australian", sub-population, and how this may relate to the very large pool of recruits produced within the large Western Pacific "population", are considerations important in the formulation of any long-term development or management policy. For example, if yellowfin tuna exploited within the AFZ are part of a free-mixing Pacific stock then quotas applied solely to AFZ catches will have little conservation value to the Australian fishery unless similar restrictions are applied outside the AFZ. To date, decisions regarding management of the east coast yellowfin fishery have been made with little or no understanding of sub-population structure, interactions between domestic and foreign fisheries (both within and outside Australian waters) or the link between spawning grounds in the Coral Sea (within the AFZ) and recruits fished along the sub-tropical east coast of Australia. The shortcomings in our current knowledge of these issues prompted participants (including representatives from industry and scientific and management agencies) in the East Coast Tuna and Billfish Research Priorities Workshop held in Sydney in March 1990 to assign the question of "stock" delineation the highest priority for future research.

In response to the requirement for accurate data, and recognizing that previous studies (e.g. Diplock and Reid in prep.; Smith et al. 1988; Anon 1989) have achieved only limited success using a variety of techniques (e.g. morphometrics, allozyme analyses, tagging), the pilot study proposed here by CSIRO seeks to test the feasibility of applying integrated data from two relatively new techniques (analysis of mitochondrial DNA and otolith microchemistry) and one established technique (allozyme electrophoresis) to the question of yellowfin sub-population structure. The need for a pilot study in this instance is clearcut; before proceeding with a large-scale project, it is essential to assess both the suitability of the two new techniques for application to yellowfin tuna and the extent of genetic and otolith microchemical variability within the study area. It would be inappropriate, for example, to continue into a second phase of the project if genetic data collected in the pilot study suggested there was little hope of differentiating between different areas of the Western Pacific. Equally important is the ability of otolith microchemical data to discriminate between fish known to originate from different spawning areas.

Incorporating the development of a genetic taxonomic key that will allow for the first time the speedy and accurate identification of larvae, post-larvae and small juveniles of yellowfin tuna, the pilot study as proposed will provide an essential baseline on which a full-scale project can be developed.

The CSIRO proposal (incorporating both the pilot and follow-up projects) was detailed by John Gunn at the East Coast Tuna and Billfish Research and Monitoring Workshop held on 15-16 March 1990 and was also discussed at ECTUNAMAC.

SECTION 5 – PROPOSAL IN DETAIL
(a) Plan of Operation
(i) Method of Procedure
Collection of samples

As a major objective of the pilot study is to examine the extent of genetic and otolith microchemical variability in yellowfin tuna known to have come from different spawning grounds, sampling efforts will concentrate on larvae, post-larvae and juveniles less than

one month old. Samples will be collected using a variety of techniques; plankton tows, dip netting with light attraction and light traps, all of which have proven successful in catching tuna larvae and small juveniles. To provide adequate geographic coverage, it is proposed that three areas will be sampled in the Western Pacific and one in the Indian Ocean. Following offers of assistance from the South Pacific Commission, samples from the Western Pacific would be collected by CSIRO staff using the SPC research vessel, the Te Tautai , as it moves throughout the region as part of its current Regional Tuna Tagging Program (RTTP). Similarly, samples from the yellowfin spawning ground in the Coral Sea off north Queensland (McPherson 1988) would be collected as part of the proposed extension of the RTTP's activities into the AFZ next October/November [1]. A sample from a location in the Indian Ocean will be collected either through collaboration with scientists in South Africa and the Seychelles or through the co-operation of Japanese scientists during the annual FRV Shoyo Maru cruise into north-west Australian waters. All material will be collected fresh and then frozen on board and airfreighted in dry ice. This is a requirement for allozyme and mtDNA analysis and precludes the use of material currently held in collections.

Development of a genetic taxonomic key

Current taxonomic keys to *Thunnus* are inadequate for the identification of larvae, post-larvae and juveniles up to 10 cm FL. As this study will concentrate on these early life history stages, it is essential that a reliable method of separating samples to species level is developed. Previous work on yellowfin (Graves et al. 1988) suggests that it is possible to distinguish this species from bigeye on the basis of the electrophoretic pattern of the muscle isozyme of glycerol-3-phosphate dehydrogenase. There also appear to be some diagnostic liver enzymes which enable adults of various tuna species to be identified (Lewis 1981), but no attempt has been made to discriminate larvae or juveniles on the basis of these characters. An enzyme-based dichotomous key to all commonly occurring tuna will be developed to enable rapid and unambiguous identification of both larvae and adults.

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[1] It is planned to utilize the Te Tautai for the collection of samples from the yellowfin spawning ground in the Coral Sea. This proposal relies on extension of the Regional Tuna Tagging Program of the SPC into the AFZ off northeastern Australia and that there will be no charter costs to be paid by CSIRO. If the Te Tautai does not carry out this work then an alternative vessel (possibly the Lady Basten) will have to be chartered and these costs added to the project budget.

Genetic variation

Both allozyme (representing nuclear DNA) and mitochondrial DNA (mtDNA) variation will be assessed. In this pilot study, a minimum of 50 specimens from each location will be analysed for allozyme variation. The preferred technique is cellulose acetate electrophoresis using Titan III plates from Helena Laboratories. This is both very sensitive and very rapid. Gene frequencies for polymorphic enzymes will be determined and differentiation among sub-populations assessed using standard chi-square approaches. Genetic variance will be partitioned among and within sub-populations.

MtDNA variation will be assessed from a minimum of 25 individuals from each location. MtDNA will be extracted, purified, digested with appropriate restriction enzymes, and the resulting fragments separated on agarose and acrylamide gels. Visualization techniques will involve both ethidium bromide and radioactive end-labelling. Statistical analysis will be very similar to the allozyme analysis.

Microchemical analysis of otoliths

25 otoliths from each sampling area will be analysed with both electron and proton microprobes using the techniques developed by CSIRO over the past four years (Gunn et al. in prep.). Chemical analyses will be performed at the CSIRO Divisions of Mineral Products (Melbourne) and Exploration and Geoscience (Sydney). Data from these analyses will allow the assessment of variation in the concentrations of 6-15 elements expected to be present in the otoliths of yellowfin tuna. Multivariate analytical techniques (discriminant functions etc.) will be used to identify and describe differences among areas.

(ii) Facilities Available

A spacious, modern and well equipped biochemical genetics laboratory has recently been established at the CSIRO Marine Laboratories in Hobart. This is currently using allozyme electrophoresis in stock discrimination studies of species in the South-East Trawl fishery, but in the next few months will be additionally equipped for mtDNA analysis. In the New Year, Dr Peter Grewe, who has extensive fish mtDNA expertise, will be joining the staff in Hobart.

The CSIRO Marine Laboratories also has a laboratory which specializes in otolith

preparation for microprobe analysis. This facility has a range of sectioning, grinding and polishing equipment specifically designed for the processing of material for microprobe analysis and is staffed by personnel with extensive experience in the field.

(b) Support Data

<u>Field collection and identification</u>

Larval tunas are currently distinguished by melanophore patterns (the distribution of dark pigment) primarily on the tail region (Collette et al. 1984; Nishikawa and Rimmer 1987). Yellowfin and bigeye tuna larvae have been separated by the presence (bigeye) or absence (yellowfin) of postanal ventral melanophores (Nishikawa and Rimmer 1987), however the reliability of these melanophores to separate these two species has been questioned (Richards and Potthoff 1974). Graves et al. (1988) have since shown using the species-specific electrophoretic pattern of glycerol-3-phosphate dehydrogenase, that a whole collection of larvae, some having none and others up to eight postanal ventral melanophores, were all yellowfin. If those larvae had been identified on the basis of melanophore patterns most would have been mis-identified as bigeye. Similar problems also occur in separating other species of tuna larvae.

As tuna larvae develop, the larval pigment patterns become obscured by general body pigment and the patterns are no longer diagnostic making it impossible to separate species (Nishikawa and Rimmer 1987). Even detailed osteological studies have failed to distinguish between yellowfin and bigeye juveniles 8 to 100 mm long (Potthoff 1974). However, as indicated above, yellowfin and bigeye larvae can be distinguished by the electrophoretic pattern of glycerol-3-phosphate dehydrogenase (Dotson and Graves 1984; Graves et al. 1988). A number of other tuna species occur in the proposed sampling areas, such as skipjack and albacore, and it will be necessary to distinguish yellowfin from these other species.

CSIRO Division of Fisheries has been investigating the distribution, ecology and sampling methods to estimate the abundance of larval tunas since 1987 (Davis and Clementson 1989; Davis et al. 1989; Davis et al. 1990a, 1990b; Jenkins and Davis 1990, Young and Davis 1990). We are experienced in the identification problems and sampling requirements for larval and post-larval tunas.

The pilot project will test a number of allozymes from muscle tissue to provide a rapid and cheap way of distinguishing between species of juvenile tuna. We will also determine whether these allozymes are effective in separating species of tuna larvae as well as juveniles. The reliability of melanophore distribution in distinguishing species of tuna larvae will also be assessed. It essential that the species identity of material be known unequivocally before mtDNA, allozyme and electron microbe analysis is carried out to determine their genetic and phenotypic origins.

Genetic variation

The only stock-based allozyme study of yellowfin tuna in Australian waters is that of Smith et al. (1988), a study based on adult fish but bedevilled by low sample sizes and by the deterioration of enzymes in some samples. The authors thus regard their results as "tentative", but nevertheless found indications of population substructuring both in Australian waters and from overseas. They considered that "further electrophoretic investigations into this species are warranted". More extensive research on adult skipjack tuna led to the description of the population structure of this resource in terms of an "isolation-by-distance" model, but it was concluded that the genetic heterogeneity within and among schools of this species meant that stock structure would have been better assessed if larvae rather than adults had been screened (Richardson 1983).

Mitochondrial DNA analysis is likely to have greater resolving power than allozyme analysis for sub-population discrimination (Avise 1987; Ward et al. 1989). This is because restriction enzyme analysis of DNA allows variability at synonymous and non-coding DNA sites to be detected, and because mtDNA evolved at 5-10 times the rate of nuclear DNA. MtDNA analysis has been carried out on some tuna species (Graves et al. 1984; Graves and Dizon 1989) but not, as yet, on yellowfin. The approach proposed here of combining both allozyme and mtDNA analysis on juvenile or larval fish will provide a very powerful test of genetic sub-population structuring.

Dr Ward, who will be responsible for the allozyme analyses, has about 20 years experience using these techniques on a wide variety of invertebrate and vertebrate taxa (including many fish species). He has more than 50 publications in this area, and, until his recent move to Australia, has worked primarily in the UK and Canada. Dr Grewe, who will be responsible for the mtDNA analyses, has about six years experience using

these and related techniques to study the sub-population structure of lake trout in Canada and the US.

Otolith analysis

The potential for the discrimination of fish stocks or sub-populations through the analysis of variability in concentrations of microconstituents of calcified tissues has been demonstrated for a number of species (Mulligan et al. 1983; Mulligan et al. 1987; Edmunds et al. 1989). The only published data on tuna are those of Calaprice (1985) who was able to discriminate between sub-populations of bluefin tuna (*Thunnus thynnus*) from different parts of the Atlantic Ocean on the basis of vertebral microchemistry. For the past four years, the CSIRO Division of Fisheries has been developing techniques for electron microprobe analysis of otoliths (Gunn et al. in prep.) and testing the potential of these for discrimination of stocks of morwong, a temperate, demersal cheilodactylid. To date these studies remain unpublished. However, there is considerable evidence to indicate that the concentrations of core elements (in particular Sr, K, Na, Cl and S) within the otolith matrix of morwong differ significantly in fish caught in different parts of south eastern Australia. Although variance among fish from any one area is, in some cases, large, the general integrity of signals from different areas allows the mapping of 'sub-population units' - it is possible to discriminate between fish caught off NSW or South Australia or Tasmania on the basis of otolith microchemistry. Differences in the chemistry of those parts of the otolith laid down in the first weeks of life also suggest that the location of spawning and larval development affects the concentration of core elements. This is of critical importance to the proposed study of yellowfin tuna, as it is the ability to differentiate between spawning areas that will determine the success or failure of this component of the work. It is the fact that a link between otolith microchemistry and spawning location has been investigated for only one species that indicates the necessity for a pilot study on yellowfin.

SECTION 6 - RESEARCH PRIORITY

The identification of the spawning population(s) supplying recruits to the east coast yellowfin fisheries and population sub-structure of yellowfin within the eastern AFZ have been identified as priority areas for research by fisheries managers and research groups.

SECTION 6 – TRANSFER OF RESULTS TO INDUSTRY

This project is a pilot study intended to provide the information needed to assess the feasibility of a larger research project to a) determine the source of yellowfin recruits to domestic and foreign commercial fisheries in eastern Australian waters and b) to discriminate yellowfin tuna sub-populations exploited within the AFZ. This information will be reported to Australian Fisheries, ECTUNAMAC and relevant Granting Agencies. This pilot study will determine whether a larger research project is practical and if so, it will provide the basic information needed to carry out such a project. It is anticipated that research in the pilot study will also result in publications in international journals.

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PROFORMA 'A'

FOR NEW APPLICATIONS

SECTION 8 - PREDICTED COMMENCEMENT & COMPLETION DATE

Commencement Date ...1.../...9.../...91...

Duration of Project .....1......
(in years)

Completion Date ..31.../...8.../...92...

SECTION 9 - REQUESTED BUDGET

Item	Requested 1991/92	Indicative 1992/93	Indicative 1993/94	Indicative 1994/95
Salaries & Wages	...50523....	....	....	....
Operating Expenses	...27700....	....	....	....
Travel Expenses	...10500....	....	....	....
Capital Items	......nil.......	....	....	....
TOTAL	$ ...88723.....	$..................	$..................	$..................

SECTION 10 - FUNDS SOUGHT FROM OTHER SOURCES

SOURCE ......Alternate..but..not..complementary$ ....

......funding..is..being..sought $ ....

......through..AFS..(ECTUNAMAC). $ ....

SECTION 11 - FINANCIAL CONTRIBUTION OF APPLICANT

Financial contribution would be the salaries of CSIRO Division of Fisheries staff and by way of the facilities listed in section 5.

Salaries

Staff	Classification	% time	Salary
Mr J.S. Gunn	CSOF5	50	22369
Dr R.D. Ward	CSOF7	30	16800
Dr P. Grewe	CSOF4	50	18195
Dr T.L.O. Davis	CSOF7	30	16478
Total			73842

On Costs

Superannuation	13587
Comcare	1846
Leave Loading	1100
Total	16533

Total **90375**

SECTION 12 – BUDGET IN DETAIL

ITEM	1990/91
Salaries & Wages	
CSOF3 12 months	32112
Superannuation	5909
Comcare	802
Leave loading	500
Marine survey allowance - (8 weeks @ $1400)	11200
Total Salaries and Wages	**50523**
Operating expenses	
Disposable laboratory items	700
Allozyme electrophoresis - plates, chemicals etc.	4000
mtDNA analysis - film, enzymes, chemicals	4000
Electron microprobe operation contract (CSIRO Div. Mineral Products)	4000
Proton microprobe operation contract (CS?? Div. Exploration Geoscience)	6000
?a mountings for otolith analysis	6000
?pling - nets, containers	1000
Domestic and international air freight of frozen material	2000
Total Operating Expenses	**27700**
Travel expenses	
New Caledonia - land-based sampling and connection with SPC tagging vessel - 2 scientists	2800
Travel allowance	800
Cairns - connection with SPC tagging vessel for 2 scientists	2400
Sydney - microprobe work (1 scientist, 2 weeks)	600
Travel allowance	1400
Melbourne - microprobe work (1 scientist, 2 weeks)	400
Travel allowance	1400
Canberra - report results to ECTUNAMAC	500
Travel allowance	200
Boat charter[2]	nil
Total Travel Expenses	**10500**
Capital Items	nil
Total Capital Items	**nil**
TOTAL REQUESTED BUDGET	**88723**
Estimated Income	nil
Total Estimated Income	**nil**

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[2] It is anticipated that there will be no charter costs - see Footnote 1.

SECTION 11- FINANCIAL CONTRIBUTION OF APPLICANT

.See..attachment..-..Section..11...

....

....

....$ ..90375.................................

SECTION 13 - ORGANIZATION

Head Responsible for Project ...Dr Peter Young...

Name of Organization .....CSIRO Marine Laboratories.................

Address
.........................Division of Fisheries...
                        Name of Department (if applicable)

.........................Castray Esplanade...

City ....Hobart............ State ..Tas.........Postcode.....7000........

Telephone ...(002) 206222..............Fax....(002) 240530. Telex..AA57182....

Postal Address: GPO Box 1538, Hobart, Tasmania, 7001

SECTION 14 - PROJECT SUPERVISOR

Name   ...Dr.T.L.O. Davis..

Address
.........................CSIRO Division of Fisheries...........................
                        Name of Department (if applicable)

.........................Castray Esplanade...

City ....Hobart.............. State ....Tas...... Post Code ..7000..........

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Continued.......PROFORMA 'A'

SECTION 15 - STAFF INVOLVED ON PROJECT

% of time:

	Quals	Role	CSIRO	FIRDC
Mr J.S. Gunn	CSOF5	Principal investigator & otolith microchemistry	50	-
Dr R.D. Ward	CSOF7	Allozyme analysis	30	-
Dr. P. Grewe	CSOF4	Mt DNA analysis	50	-
Dr T.L.O. Davis	CSOF7	Identification & morphology	30	-
CSOF3 (Laboratory assistant - to be appointed)			-	100

SECTION 16 - ADMINISTRATIVE CONTACT

Name   Mr Peter Green

Address

CSIRO Marine Laboratories
Name of Department (if applicable)

Castray Esplanade

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