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Determination of the stock relationships of orange roughy between the Lord Howe Rise and adjacent New Zealand areas

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Final Research Report

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7. Executive Summary

Three molecular methods were used to estimate genetic differences within and between samples of orange roughy from the southwest and northwest Challenger Plateau and the Lord Howe Rise. Tissue samples were available from previous genetic analyses and two additional samples were collected during 1998. Genetic differentiation between the southwest and northwest Challenger was confirmed with the allozyme marker *Idh-2**. A mitochondrial DNA marker distinguished samples from the Lord Howe Rise and Challenger Plateau, but did not separate samples from the southwest and northwest Challenger. Two new microsatellite DNA markers showed high levels of genetic variation but no significant regional differentiation.

8. Objectives

To determine stock relationships between orange roughy inside the EEZ and adjacent areas outside the EEZ.

Objective 3. To determine the stock relationships of orange roughy between the Lord Howe Rise and adjacent New Zealand areas from samples collected during 1996–97.

9. Methods

The objective is divided into three activities: collection of tissue samples, laboratory analyses of tissue samples and data analyses. Previous genetic analyses of orange roughy tissue samples from the Tasman Sea had found one allozyme marker, isocitrate dehydrogenase *Idh-2**, that revealed significant heterogeneity among samples from the Challenger Plateau and Lord Howe Rise (Smith 1997a). However the *Idh-2** marker has shown significant temporal variation in other regions of the New Zealand EEZ (Smith 1997b, Smith and Benson 1997). Therefore additional tissue samples were collected to test for temporal variation in the Tasman Sea. Previous genetic analyses using mitochondrial DNA markers revealed unique restriction fragments on the Challenger Plateau in 1988 and 1989 (Smith et al 1996); this method was tested in samples collected between 1996–98.

A new, and potentially more sensitive, DNA technique has become available for testing genetic variation in fish populations based on the amplification of microsatellite DNA (Wright and Bentzen 1994). Two pairs of microsatellite primers have been developed specifically for orange roughy by C. Oke, La Trobe University; and these were tested on the New Zealand samples.

Samples

Tissue samples have been collected through earlier Ministry of Fisheries programmes, in particular Project DEOR13, Genetic stock discrimination of orange roughy in the Tasman Sea (Smith 1997a). These tissue samples had been tested for allozyme markers and mtDNA fragments of an amplified region of the mtDNA (Smith 1997a), but not the restriction fragments of the whole mtDNA genome (Smith et al 1996). The frozen tissues were available for analyses of additional genetic markers for this project (Table 1).

Additional tissue samples of orange roughy were collected through the Ministry of Fisheries Observer Programme on vessels operating outside the New Zealand EEZ on the northwest Challenger Plateau and Lord Howe Rise (Table 2). Observers were briefed prior to departure and given a set of sampling instructions for removal and storage of tissues from orange roughy. Ideally tissue samples would be taken from 100 fish, based on 4 tows each of 25 fish and include approximately equal number of males and females. Heart, liver, and muscle tissues were stored in liquid nitrogen at sea and transferred to -70°C freezers in the laboratory. Length, sex and position data were recorded for each fish sampled for tissues.

Allozymes: laboratory analyses

One marker, *Idh-2**, was tested in heart tissue. Electrophoretic procedures followed those described in Smith (1986), except that BDH (British Drug House Chemicals Ltd, Poole) starch was substituted for Electrostarch (Electrostarch Company, USA). Small pieces of heart tissue (0.5g) were homogenised in an equal volume of 1% triton in deionised water, centrifuged at 8 000g for 5 min and the supernatant applied to filter paper wicks and inserted into starch gels made up in a phosphate citrate buffer system (Selander et al 1971). Gels were run for five hours and then stained for *IDH** following standard procedures. Genetic analyses were performed using the BIOSYS-1 programme (Swofford and Selander, 1981).

Allozymes: statistical analyses

For each site genotype frequencies were tested for conformance to Hardy-Weinberg expectations. χ^2 tests were carried out on pooled genotypes to reduce the number of classes with expected values <5. Allele frequencies were tested for heterogeneity within and among areas with contingency χ^2 tests; samples included those collected during 1998 and those previously analysed from the Challenger-Lord Howe Rise and reported in Smith (1997a). To test for geographic structure, contingency χ^2 tests were undertaken within and among sites of neighbouring populations. Probability levels were modified by the Bonferroni procedure for multiple tests after Rice (1989). The proportion of *Idh-2** variation due to differentiation among populations was estimated with Nei's gene-diversity statistic, G_{ST} (Nei, 1973). G_{ST} is equal to $(H_T - H_S)/H_T$ where H_T is the genetic diversity in all populations and H_S is the mean genetic diversity per population, calculated from the expected heterozygosities. Sampling error will produce differences in allele frequencies, even when samples are drawn from the same population, therefore a randomisation test was used to test for differences due to sampling error (Elliott and Ward, 1992). One thousand randomisations were used and the probability was estimated from the number of randomisations that were equal to or greater than the observed G_{ST}.

Mitochondrial DNA: laboratory analyses

Mitochondrial DNA extraction methods followed those of Chapman and Powers (1984). Approximately 0.5 g liver was homogenised in TEK buffer pH7.5, and cell debris removed by gentle centrifugation. The mtDNA was pelleted by centrifugation at 18 000g forl hr, and the pellet lysed in 1% non-idet. The DNA was extracted with a phenol chloroform protocol and precipitated in ethanol at 4^0C; the pellet was dried and re-suspended in 20µl water. A 10 µl sub-sample was digested with the six-base restriction enzyme *Bgl*1 following manufacturer's instructions. The restriction fragments were separated in 1% agarose gels in a TEB buffer system, stained with ethidium bromide and viewed with a UV transilluminator.

Mitochondrial DNA: statistical analyses

The different restriction fragments were given an alphabetic code with C for the most common pattern, following Smith et al (1996). Heterogeneity of haplotype frequencies was tested with a Monte Carlo randomisation test described by Roff and Bentzen (1989) using the REAP package (McElroy et al 1992). This method overcomes the problem of observed haplotypes at low frequency by comparing χ^2 values obtained in many (1 000) random rearrangements of the data. Probability levels were estimated from the number of randomisations that were equal to or greater than the observed χ^2 value.

Microsatellite DNA: laboratory analyses

Amplification reactions were performed in 50 µl volumes in a Perkin Elmer Cetus DNA thermocycler. Two primer pairs, OR2A and OR40, developed by Oke, La Trobe University (pers comm), were purchased from Operon Technologies Inc. Following successful amplification and separation of microsatellite PCR products in poly-acrylamide gels, an additional primer from each pair was purchased as a fluorescent-labelled primer to allow analysis of amplified product in an Perkin Elmer 373 automated DNA sequencer. DNA samples were amplified with labelled primers, and the PCR products tested in agarose gels. PCR products from the same fish, but amplified with different primer pairs, were pooled in one reaction tube and run in one

gel lane, along with a labelled DNA standard, in the automatic sequencer. Fluorescent labelled products were quantified in base pairs by the amount of fluorescence using the GENESCAN software from Perkin-Elmer Corporation.

Microsatellite DNA: statistical analyses

PCR fragments separated by two, or more, base pairs were scored as alleles. Allele frequencies were determined by direct counting of homozygous and heterozygous individuals. Expected heterozygosities and χ^2 tests for deviation of genotype frequencies from Hardy-Weinberg equilibrium were calculated using Fisher's exact test with the BIOSYS-1 programme (Swofford and Selander 1981). Alleles were binned to produce 10, or less, alleles per sample by combining alleles separated by 2 base pairs. Allele frequency comparisons between population samples were made with a 2 x n χ^2 contingency test on binned alleles. Within site heterogeneity was tested by comparing samples collected in different years within areas; between area heterogeneity was tested by comparing all samples. In addition allele frequency heterogeneity among samples was tested with the Monte Carlo randomisation approach of Roff and Bentzen (1989). This method is similar to the χ^2 test, but the test distribution is generated by randomising the original matricies to overcome the problem of large numbers of rare alleles. The randomisation tests were made on all alleles.

The degree of population sub division, the proportion of total genetic variation that occurs between populations, was estimated with two methods.

- Wright's F statistics (Wright 1965), in particular F_{ST} values, which are based on an infinite allele model, were calculated from heterozygosites of binned alleles using the BIOSYS-1 programme (Swofford and Selander 1989); significance levels were tested after Workman and Niswander (1970). F_{ST} values were also calculated by the variance method (= θ), based on all alleles, following Weir and Cockerham (1984). Concern has been expressed that microsatellite mutations contravene the assumptions on which F_{ST} values are based, and F_{ST} measures will tend to underestimate the level of genetic differentiation. Microsatellite loci deviate from an infinite allele model because many mutations are likely to produce allelic states that are the same size as existing alleles, and are not distinguished by electrophoretic methods.
- A revised statistic, R_{ST} (Slatkin 1995), which is analagous to Wright's F_{ST} but takes into account the mutation rates at microsatellite loci, was used to estimate genetic differentiation using the RST-CALC programme (Goodman 1997). The programme uses a permutation method to determine if observed P values are significantly different from zero. Both F_{ST} (as θ) and R_{ST} were also estimated with the RST programme of Ruzzante (Ruzzante et al 1996); signifance values for tests were determined by Monte Carlo simulations.

10. Results

Sample collection

Observers were briefed and liquid nitrogen cylinders and sampling kits loaded onto four vessel trips to collect material from the Lord Howe Rise and northwest Challenger Plateau fisheries. On two occasions the vessel diverted to other fishing grounds while at sea; samples were collected from the South Tasman Rise and the Louisville Ridge (Table 2). On the third trip no samples were collected due to a vessel breakdown on the first day of fishing. On the fourth trip samples were collected from the Northwest Challenger and Southwest Challenger outside the EEZ in May-June 1998 (Table 2). There has been little fishing activity by New Zealand vessels on the Lord Howe Rise. The fishing vessel *Arrow* fished on the Lord Howe Rise in August-September 1998 and caught no orange roughy, but did catch orange roughy on the northwest Challenger. Tissue samples were collected from a further 50 fish, but were not included in the laboratory analyses due to late arrival (25 September 1998) in Wellington. The samples available from previous Ministry of Fisheries projects, and included in this project for allozyme analyses are listed in Table 1; samples used for mitochondrial and microsatellite DNA analyses are marked with an asterisk.

Table 1. Sample locations and dates for *Hoplostethus atlanticus* tissue samples from the Challenger Plateau and Lord Howe Rise used in the allozyme analyses; samples used for the mitochondrial and microsatellite DNA analyses are marked *. Locations are given as the approximate position at the start of a tow.

Site/Year	Lat (S)	Long (E)	Date	Vessel	No Fish
LordHowe96*	35° 42'	165° 59'	24-25.10.96	*Arrow*	100
NWChallenger96*	37° 18'	167° 21'	16-26.6.96	*Arrow*	200
NWChallenger97	37° 13'	167° 20'	28.6.97	*Baldur*	25
SWChallenger96*	39° 57'	168° 00'	5-10.7.96	*Baldur*	100
SWChallenger97a	40° 04'	167° 58'	25.06.97	*Seafire*	100
SWChallenger97b	40° 11'	167° 54'	2-9.6.97	*Amaltal Endeavour*	100

Table 2. Observer coverage and *Hoplostethus atlanticus* tissue sample collection for the Lord Howe Rise and Challenger Plateau 1997-98.

Area (activity)	Date	Vessel	Number of fish
(divert to Tasman Rise)	Sep 1997	*Atlantic Elizabeth*	100
(break down)	Jan 1998	*Atlantic Elizabeth*	0
(divert to Louisville)	Jan 1998	*Atlantic Elizabeth*	100
40°S 167° 50E	May 1998	*Sea Ranger*	100
38°12'S 167°14'E	June 1998	*Sea Ranger*	25

Allozymes

Genotype numbers for the 1998 samples are given in Table 3. There is no significant departure from Hardy-Weinberg equilibrium (SW98 $\chi^2 = 3.18$, $P = 0.07$; NW98 $\chi^2 = 0.10$, $P = 0.75$). There is significant heterogeneity in the total *Idh-2** data indicating that the samples have been taken from more than one genetic group (Table 4). The data were broken down into regional samples to test for spatial heterogeneity. Rare genotypes were lumped to reduce the data to a biallelic system. Removing the Lord Howe Rise sample, then the heterogeneity persists in the Tasman Sea data set (Table 4: SW&NW).

Replicate samples were collected from the southwest and northwest Challenger Plateau in 1996, 1997, and 1998. There is no significant heterogeneity among the southwest Challenger samples from 1996/97/98 (Table 4); likewise there is no significant heterogeneity among the northwest Challenger samples from 1996/97/98, although the 1997 and 1998 samples were small (N < 25). There is a significant heterogeneity between the northwest and southwest Challenger Plateau samples collected over 1996/97/98. However there is no significant difference among the 1998 samples from the southwest and northwest (Table 4), due to the relatively small size of the northwest 1998 sample (n=25). The difference between the Lord Howe Rise and northwest Challenger Plateau is maintained with the northwest Challenger Plateau sample from 1998 (Table 4).

Table 3. *Idh-2** genotypes in population samples of *Hoplostethus atlanticus* collected on the Challenger Plateau during 1998.

Site	Genotypes			
	AA	**AB**	**BB**	**BE**
Northwest	2	11	10	0
Southwest	18	37	42	1

Table 4. *Idh-2** diversity in *Hoplostethus atlanticus* for lumped alleles, measured with a χ^2 contingency test and Nei's gene diversity (G_{ST}). Data include all sites, all sites minus the Lord Howe Rise (LH), Southwest Challenger (SW), Northwest Challenger (NW), Northwest Challenger and Lord Howe, and the 1998 samples from Southwest and Northwest Challenger. * significant at the 5% level using Bonferroni modified *P*.

Sites	χ^2	**d.f**	***P***	G_{ST}	***P***	G_{STnull}
all	35.1	7	<0.001*	0.045	<0.001*	0.008
SW&NW	31.7	6	<0.001*	0.046	0.001*	0.009
SW	2.5	3	0.483	0.004	0.439	0.004
NW	4.7	2	0.096	0.032	0.063	0.011
NW&LH	23.2	3	<0.001*	0.057	0.001*	0.01
1998	0.4	1	0.515	0.003	0.502	0.007

Mitochondrial DNA

The numbers of observed *Bgl*1haplotypes are summarised in Table 5. There is a significant heterogeneity in the total data (χ^2 = 15.35, P= 0.02), but not in the southwest (χ^2 = 1.86, P = 0.41), the northwest (χ^2 = 0.09, P = 0.59), or the combined northwest and southwest (χ^2 = 7.42, P = 0.26).

Table 5. Mitochondrial DNA *Bgl* 1 haplotypes in *Hoplostethus atlanticus* from the Challenger Plateau and Lord Howe Rise.

Area	***Bgl* 1 haplotypes**		
	A	**B**	**C**
southwest 1996	0	6	42
southwest 1998	1	8	34
northwest 1996	0	3	44
northwest 1998	0	1	21
Lord Howe Rise 1996	0	0	46

Microsatellite DNA

Allele frequencies for the two microsatellite loci for five population samples are shown in Table 6. Both loci are strongly polymorphic with between 7–10 and 12–17 alleles. The genetic data for each sample are summarised in Table 7. Average heterozygosities ranged from 0.559 (Lord Howe Rise) to 0.824 (Southwest Challenger 1996). There was a significant excess of homozygotes in 3 samples at the OR2A locus (Lord Howe Rise 1996, P =0.025; northwest Challenger 1998, P = 0.015; and southwest Challenger 1998, P = 0.003). A comparison of allele frequencies found no significant heterogeneity among all samples for the two loci. However there was a marginally significant heterogeneity among the two samples from the northwest Challenger at the OR2A locus (P = 0.03, Bonferroni modified P = 0.02 for multiple tests); χ^2 values for the within and between area comparisons are given in Table 8. The 1998 sample from the northwest Challenger consisted of only 25 fish and so the within area heterogeneity may be generated by sampling error at a highly polymorphic locus. Likewise the χ^2 randomisation test, based on all alleles, found a significant heterogeneity within the northwest samples at the OR2A locus, but all other tests within and among regions were none significant (Table 9).

Table 6. Microsatellite allele frequencies in 5 population samples of *Hoplostethus atlanticus* from the Challenger Plateau and Lord Howe Rise. N = northwest Challenger; S = southwest Challenger; L= Lord Howe Rise; 96, 98 year of sampling.

Locus	**Population**				
allele	**N96**	**S96**	**L96**	**N98**	**S98**
OR2A					
(N)	48	45	46	20	46
A	.115	.222	.283	.100	.163
B	.250	.189	.207	.275	.217
C	.104	.122	.098	.200	.130
D	.177	.200	.174	.150	.130
E	.073	.033	.033	.025	.065
F	.188	.144	.163	.125	.163
G	.083	.056	.022	.000	.098
H	.010	.033	.022	.125	.033
OR40					
(N)	42	46	46	22	46
A	.095	.163	.144	.159	.196
B	.143	.065	.078	.045	.065
C	.143	.152	.189	.091	.141
D	.012	.076	.067	.068	.054
E	.036	.033	.033	.091	.065
F	.202	.130	.133	.114	.152
G	.048	.076	.022	.068	.054
H	.226	.217	.222	.273	.207
I	.036	.054	.078	.023	.022
J	.060	.033	.033	.068	.043

The two measures of population subdivision also showed no evidence for regional differentiation: F_{ST} and R_{ST} produced similar non-significant levels of genetic differentiation across all samples and both loci (Table 10).

Table 7. Genetic variability for two microsatellite loci in 5 population samples of *Hoplostethus atlanticus* from the Lord Howe Rise (LH96), northwest Challenger Plateau (NW96, NW98) and southwest Challenger Plateau (SW96, SW98). Number of fish scored, total number of alleles observed, size range of the alleles in base-pairs, number of heterozygotes observed, and number of heterozygotes expected assuming Hardy-Weinberg equilibrium. * significant excess of homozygotes at the 5% level with a modified *P* for multiple tests.

Parameter	Area and year				
	NW96	SW96	LH96	NW98	SW98
OR2A					
No. fish	48	45	46	20	46
No. alleles	9	8	8	7	9
allele size range	170-194	170-198	170-198	170-198	170-198
Hets observed	36	36	29	6	26
Hets expected	40	38	37*	17*	39*
OR40					
No. fish	42	46	45	22	46
No. alleles	16	15	14	12	17
allele size range	262-316	262-316	262-316	262-312	262-316
Hets observed	35	39	38	18	38
Hets expected	36	40	39	19	39

Table 8. Allele frequency comparison χ^2 values for within and between area samples of *Hoplostethus atlanticus* from the Lord Howe Rise, northwest Challenger (NW) and southwest Challenger (SW). Allele frequencies are based on binned alleles. *d.f.* = degrees of freedom, *P* = probability; *P* <0.02 to be significant at the 5% level using Bonferroni modified P.

Sample	Locus								
	OR2A			OR40			SUM		
	χ^2	*d.f.*	*P*	χ^2	*d.f.*	*P*	χ^2	*d.f.*	*P*
NW	15.70	7	0.03	10.66	9	0.30	26.36	16	0.05
SW	4.47	7	0.72	3.58	9	0.94	8.05	16	0.95
All	38.9	28	0.08	29.0	36	0.79	67.9	64	0.34

Table 9. Heterogeneity of *Hoplostethus atlanticus* allele frequencies from the Challenger Plateau (NW and SW) and Lord Howe Rise, based on a randomisation test of all alleles. * significant at the 5% level with a Bonferroni modified *P* for multiple tests.

Area	OR2A			OR40		
	No. alleles	χ^2	*P*	No. alleles	χ^2	*P*
NW	9	15.63	0.02*	17	13.2	0.64
SW	9	6.76	0.59	17	11.13	0.82
ALL	10	50.25	0.05	17	63.4	0.51

Table 10. Genetic differentiation measures for population samples of *Hoplostethus atlanticus* from the northwest and southwest Challenger, and all Tasman Sea samples, based on all alleles. NA not applicable due to negative value.

Locus	northwest Challenger		southwest Challenger		Sum	
	F_{ST}/R_{ST}	*P*	F_{ST}/R_{ST}	*P*	F_{ST}/R_{ST}	*P*
F_{ST}						
OR2A	-0.0007	NA	0.006	0.251	0.0002	0.412
OR40	-0.004	NA	-0.006	NA	-0.004	NA
SUM	-0.003	NA	0.0002	0.386	-0.002	NA
R_{ST}						
OR2A	-0.018	NA	0.009	0.226	0.007	0.256
OR40	-0.017	NA	0.010	0.166	-0.009	NA
SUM	-0.018	NA	0.009	0.194	-0.001	NA

11. Conclusions

1. Orange roughy catch rates have been very low on the Lord Howe Rise and there is currently little commercial fishing by New Zealand vessels in this area.

2. The allozyme data support the earlier finding (project DEOR13) that the fish on the Lord Howe Rise are a separate stock to those on the northwest Challenger Plateau which in turn are a separate stock to fish on the southwest Challenger Plateau.

3. The mitochondrial DNA data on *Bgl*1 restriction haplotypes show a significant difference between samples from the Lord Howe Rise and the northwest Challenger Plateau, but no differences between samples from the southwest and northwest Challenger Plateau.

4. The two microsatellite DNA markers show no significant differences within and among samples from the southwest Challenger, northwest Challenger Plateau and the Lord Howe Rise.

12. Publications

None

13. Data storage

Electronic copy of report held at NIWA Greta Point. Data from the Atlantic Elizabeth trip to the South Tasman Rise have been stored on the Observer data base. Allozyme data on BIOSYS programme at NIWA. Mitochondrial DNA data on REAP programme at NIWA. Microsatellite DNA data on RST programme at NIWA.

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