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Determination of the stock relationships between orange roughy on the northeast Chatham Rise and the Louisville Ridge

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Objective 4

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7. Executive summary

Two genetic methods, mitochondrial DNA and microsatellite DNA, were used to test genetic relationships among orange roughy samples from the northeast Chatham Rise and the Louisville Ridge. Analyses were based on tissue samples collected between 1994–1998. A restriction enzyme analysis of the D-loop region of mitochondrial DNA found 11 haplotypes. There was a significant heterogeneity in the total data, produced for the most part by the 1994 sample from the Box; removal of this one sample resulted in no significant genetic differentiation among samples from the north Chatham Rise and the Louisville Ridge. The distribution of mitochondrial DNA haplotypes does not distinguish between stocks from the Chatham Rise and the Louisville Ridge, yet the same haplotype markers applied in other regions have shown significant genetic differentiation. Two microsatellite loci were tested on two sets of samples; there were no significant differences between the 1995 and 1998 samples from the Louisville Ridge and no differences between 1995 and 1998 samples from the Box. However there was significant heterogeneity in the combined Louisville/Box data at one microsatellite locus, indicating that the samples have been taken from different genetic groups.

8. Objectives

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

Objective 4. To collect the required samples and determine the stock relationships of orange roughy between the Louisville Ridge and the Chatham Rise from samples collected during 1997–98.

9. Methods

Two genetic methods were tested on the samples: mitochondrial (mt) DNA and microsatellite DNA. Previous analyses of allozymes, applying 8 polymorphic loci, had shown no significant differences between samples from the Arrow Plateau and the Louisville Ridge (Smith and Benson 1997). Analyses of mtDNA restriction fragment length polymorphisms had shown significant differences between samples from the Ritchie Hills and Waitaki, and between Waitaki and Puysegur (Smith *et.al.* 1997), but not among samples along the north Chatham Rise (Smith 1997). Therefore this method was applied to two samples from the Louisville Ridge for comparison with data from the Chatham Rise (Smith 1997, Smith *et.al.* 1997).

Microsatellite DNA is a relatively new genetic method applied to stock discrimination of marine fishes. High levels of genetic variation and large numbers of alleles have been found with microsatellite DNA compared with other molecular methods (eg Bentzen *et. al.* 1996, Ruzzante *et. al.* 1996). The high mutation rate, compared with allozymes, makes microsatellites potentially useful markers for demographic studies. Specific microsatellite primers have been produced for orange roughy by Oke, La Trobe University (pers comm) and two pairs of primers were used to amplify regions of the nuclear DNA. No funding was allocated to the development of additional microsatellite primers.

Samples

Frozen tissue samples were available from previous collections from the northeast Chatham Rise, and the Louisville Ridge obtained through the Ministry of Fisheries and the Orange Roughy Management Company orange roughy genetic stock discrimination projects during 1993–96 (see summaries in Smith 1997, Smith and Benson 1997, Smith *et.al.* 1997). Two additional samples were collected opportunistically through the Ministry of Fisheries Observer programme on the Louisville Ridge and through the NIWA RV *Tangaroa* voyage to the Chatham Rise during 1998 (Table 1).

Table 1. Sample locations for orange roughy tissue samples collected between 1994–98 and used for mitochondrial DNA analyses; samples used for microsatellite DNA analyses are marked with an asterisk. Locations are given as the approximate position at the start of a tow.

Location	Vessel	Date (month/year)	Latitude	Longitude
Graveyard 180	*Tangaroa*	7.96	42°47'S	179°58'E
North CRise178W	*Tangaroa*	7.96	42°45'S	178°45'W
Box	*Tangaroa*	7.94	42°47'S	177°00'W
Box*	*Tangaroa*	7.95	4 week samples	
Box	*Tangaroa*	7.96	42°50'S	177°10'W
Box*	*Tangaroa*	7.98	42°50'S	177°00'W
Louisville*	*San Rangitoto*	3.95	40°54'S	165°04'W
Louisville*	*Atlantic Elizabeth*	1.98	41°52'S	163°50'W

Laboratory analyses: DNA extraction

DNA was extracted from liver tissue of 50 orange roughy from each site. For each liver sample approximately 50 mg of tissue was homogenised in 1 ml DNAzol (Gibco BRL Life technologies, Grand Island New York), centrifuged at 10 000g, and the supernatant re-extracted with phenol, followed by chloroform-isoamyl alcohol. The DNA was precipitated in 100% ethanol at -20^{0}C overnight. The DNA was washed in 90% ethanol, air dried and re-suspended in 40µl de-ionised water.

Laboratory analyses: mitochondrial DNA amplification and restriction enzyme digestion

Amplification reactions were performed in 50 µl volumes in a Perkin Elmer Cetus DNA thermocycler, protocols followed those of Palumbi *et. al.* (1991). A region of the D-loop was amplified with the primers: 5'-ATAGTGGGGTATCTAATCCCA-3' and 5'-RCRCCCAAAGCTRRRRTTCTA-3' (Palumbi *et. al.* 1991). Twelve restriction endonucleases recognising 4-base sites (*Bfa* I, *BstU* I, *Cfo* I, *Hae* III, *Hpa* II, *Mse* I, *Msp* I, *Nla* III, *Rsa* I, *Sal* I, *Sau* 3A, and *Taq* I) were used to digest the D-loop primer amplification products in 24 fish from Louisville 1998. Three restriction enzymes (*BstU* I, *Cfo* I, and *Nla* III) that showed polymorphisms were used to test 50 fish from the two Louisville samples from 1995 and 1998. The amplified and digested DNA fragments were separated in 1.4% agarose gels and detected with ethidium bromide under a UV light (312nm).

Statistical analyses: mitochondrial DNA

Data were collected as individual haplotypes and analysed along with data from previous analyses of this mtDNA region for samples collected along the north Chatham Rise under the Ministry of Fisheries Project FBOR01 (Smith 1997, Smith *et.al.* 1997). Heterogeneity in haplotype frequencies in the total data was tested by the χ^2 randomisation test described by Roff and Bentzen (1989) using the REAP package (McElroy et. al. 1992). This method overcomes the problem of a large number of observed haplotypes at low frequency, by comparing χ^2 values in 1000 random rearrangements of the data. Probabilities were estimated from the number of randomisations that were equal to or greater than the observed χ^2 value.

The proportion of haplotype variation due to differentiation among populations was estimated with Nei's gene-diversity statistic, G_{ST} (Nei, 1973), which is a multi-allele estimator of Wright's F_{ST} statistics (Wright 1951). G_{ST} is equal to $(H_T - H_S)/H_T$ where H_T is the total genetic diversity of all populations and H_S is the mean genetic diversity per population, calculated from the average expected heterozygosities. Sampling error will produce differences in haplotype 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}.

Laboratory analyses: microsatellite DNA

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.

Statistical analyses: microsatellite DNA

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 bewteen populations, was estimated with two methods.

1. Wright's F statistics (Wright 1951), 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 1981); 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.
2. 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 detemine 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

Mitochondrial DNA

The estimated size of the PCR amplified D-loop was 1500 base pairs. Three restriction enzymes, *BstU* I, *Cfo* I, and *Nla* III, produced two or more fragment patterns with the D-loop primers in the first 24 fish tested from Louisville Ridge, and were tested in two samples of 50 fish from Louisville. The Louisville data were compared with data from the Chatham Rise, and the numbers of haplotypes observed at each site are shown in Table 3. Eleven haplotypes were observed in the total sample, although only two of these haplotypes were common ($p>0.05$).

Table 3. Mitochondrial DNA haplotypes in population samples of orange roughy from the Chatham Rise. Gra96 = Graveyard; Est96 = 178^{0}W; Box94, Box95, Box96, Box98 = Spawning Box; and Lou95, Lou98 = the Louisville Ridge, with year of sampling. Haplotype = mtDNA haplotypes based on the 3 restriction enzymes *BstU* I, *Cfo* I, *Nla* III.

Haplotype	Gra96	Est96	Box94	Box95	Box96	Box 98	Lou95	Lou98
AAA	18	14	19	18	24	26	24	21
BBA	20	22	18	18	12	20	19	20
ABA	0	1	4	0	0	1	1	0
BAA	0	0	6	0	0	1	1	0
BBC	0	0	0	1	0	0	0	0
CBA	3	0	0	0	0	0	0	0
AAB	5	6	1	1	2	0	1	2
ACA	1	0	0	1	0	0	0	0
AAC	1	0	0	0	0	0	0	0
BDA	0	1	0	0	0	1	0	0
BBB	0	0	0	0	1	0	0	1

Analyses are summarised in Table 4. There is a significant heterogeneity in the total data with the χ^2 randomisation test showing that none of the 1 000 randomisations exceeded the original χ^2 value ($P < 0.001$, Table 4). There is no significant heterogeneity among the two Louisville Ridge samples (Table 4), but there is significant heterogeneity among the four replicate samples from the Box (Table 4). The 1994 Box sample contains two haplotypes that appeared in 4 and 6 fish but were rare (1 fish) or absent in all other samples (Table 3). Removing the 1994 Box sample from the data set then there is no significant heterogeneity among the samples (Table 4).

The G_{ST} analyses show no significant differentiation among the samples. G_{ST} was estimated to be 0.027 (Table 4), which is not significantly greater than that due to sampling error, and indicates that around only 3% of the observed genetic variation is due to differences among populations. The G_{ST} analyses, which divide total variation into within and among samples, are less affected by rare haplotypes which contribute little to population differentiation. Most of the heterogeneity detected with the χ^2 randomisation test is produced by rare haplotypes in the Box 1994 sample (Table 3).

Table 4. Orange roughy mtDNA diversity at all sites, measured with a χ^2 randomisation test and Nei's gene diversity (G_{ST}). * Significant at the 5% level, with a Bonferroni modified P for multiple tests.

Area-year	χ^2	P	G_{ST}	P	G_{STnull}
all 94-98	115.43	<0.001*	0.027	0.167	0.019
Chatham Rise	90.53	0.001*	0.034	0.123	0.023
Box 94,95,96,98	38.75	0.007*	0.030	0.135	0.017
Louisville 95,98	3.52	0.842	0.003	0.786	0.010
Box 95,96,98	16.05	0.437	0.018	0.334	0.016
all less Box 94	71.52	0.098	0.026	0.212	0.019

Microsatellite DNA

Allele frequencies, of the binned alleles, for the two microsatellite loci in four population samples are shown in Table 5. Both loci are strongly polymorphic with total numbers of alleles between 10–13 and 12–17. The genetic data for each sample are summarised in Table 6. Average heterozygosities ranged from 0.576 (Louisville 1998) to 0.787 (Box 1995). There was a significant excess of homozygotes in 2 samples at the OR2A locus (Louisville 1998, P <0.001; and Box 1998, P = 0.007) and a non-significant excess for the Louisville 1998 sample (P = 0.056). A comparison of binned allele frequencies found no significant heterogeneity within and between areas for the two loci; χ^2values for the within and between area comparisons are given in Table 7. However the randomisation test, based on all alleles, found a significant heterogeneity at OR40, but not OR2A in the total data (Table 8). This heterogeneity is not apparent within Louisville or within the Box (Table 8) and thus represents a significant genetic difference between the two areas.

Table 5. Frequencies of binned alleles at two microsatellite loci in four population samples of orange roughy from the Louisville Ridge (Lou95, Lou98) and the Box (Box95, Box98).

Locus	Population			
	Lou95	Lou98	Box95	Box98
OR2A				
(No.)	*50*	*46*	*47*	*48*
A	.340	.228	.213	.167
B	.220	.217	.245	.198
C	.090	.098	.085	.083
D	.070	.087	.138	.167
E	.100	.054	.085	.042
F	.100	.228	.128	.250
G	.060	.065	.074	.042
H	.020	.022	.021	.042
I	.000	.000	.011	.010
OR40				
(No.)	*45*	*46*	*47*	*43*
A	.100	.196	.149	.163
B	.056	.087	.117	.128
C	.200	.174	.117	.151
D	.033	.033	.011	.058
E	.056	.033	.053	.070
F	.200	.152	.149	.116
G	.000	.000	.032	.047
H	.233	.239	.223	.186
I	.044	.054	.138	.058
J	.078	.033	.011	.023

Table 6. Genetic variability for two microsatellite loci in four population samples of orange roughy from the Louisville Ridge and the Box. Number of fish scored, 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 at the 5% level with a modified P for multiple tests.

Parameter	Louisville 95	Area and Louisville 98	Year Box 95	Box98
OR2A				
No. fish	50	46	47	48
No. alleles	13	10	11	12
allele size range	168-198	170-200	170-198	170-198
Hets observed	24	20	37	28
Hets expected	40	38*	38	40*
OR40				
No. fish	45	46	47	43
No. alleles	13	12	12	15
allele size range	274-316	274-316	274-316	274-316
Hets observed	38	33	37	36
Hets expected	38	38	40	37

Table 7. Allele frequency comparison χ^2 values (based on binned alleles) for within and between area samples of orange roughy from the Louisville Ridge and the Box. *d.f.* = degrees of freedom, *P* = probability.

Sample	Locus								
	OR2A			OR40			SUM		
	χ^2	*d.f.*	*P*	χ^2	*d.f.*	*P*	χ^2	*d.f.*	*P*
Louisville	8.48	7	0.29	6.52	8	0.56	15.01	15	0.45
Box	7.93	8	0.44	7.96	9	0.54	15.89	17	0.53
All	28.43	24	0.24	35.94	27	0.11	64.38	51	0.09

Table 8. Heterogeneity χ^2 tests on orange roughy allele frequencies from the Louisville Ridge and the Box, based on a randomisation test of all alleles. * significant test result.

Area	OR2A			OR40		
	No. alleles	χ^2	*P*	No. alleles	χ^2	*P*
Louisville	14	8.77	0.83	16	22.49	0.09
Box	14	11.79	0.57	17	16.81	0.39
All	14	35.73	0.62	17	99.55	<0.001*

Population differentiation

F_{ST} estimates based on binned alleles and four population samples were 0.013 for OR2A (P >0.10) and 0.008 for OR40 (P >0.30) with a mean of 0.011 (P >0.50), which are non- significant. F_{ST} estimates based on all alleles are summarised in Table 9. There is no significant heterogeneity within and among areas for the OR2A locus. However there is a significant heterogeneity among, but not within areas for the OR40 locus (Table 9), and for the combined Louisville samples compared with the combined Box samples (OR40 F_{ST} = 0.01 P <0.01; combined OR2A and OR40 F_{ST} = 0.006, P = 0.02). R_{ST} estimates based on all alleles are non-significant within and among areas for both loci (Table 9).

The apparent discrepancy between F_{ST} and R_{ST} is surprising. In general R_{ST} is better suited than F_{ST} for analysing microsatellite loci and yields less biased estimates of demographic structure than F_{ST} (Slatkin 1995). F_{ST} estimates should underestimate the level of divergence compared to R_{ST} when the divergence time is long, and both mutation and drift contribute to population differentiation (Slatkin 1995). Similar differences between F_{ST} and R_{ST}, with significant F_{ST} but not R_{ST} values, were reported for Atlantic cod *Gadus morhua* (Bentzen et al 1996). A discrepancy between F_{ST} and R_{ST} could indicate a relatively short time since divergence, although this intuitively seems unlikely for a marine fish with large population sizes and for which differences due to drift alone would take thousands of years.

The lack of population genetic differentiation with the R_{ST} analyses (Table 9) also contrasts with the allele frequency differences detected with the randomisation test (Table 8). Thus it is possible that there is some deviation from the stepwise model underlying the R_{ST} as suggested by Goodman (1998).

The current data on orange roughy based on allele frequencies and F_{ST} demonstrate a significant genetic difference among the samples from the Box and the Louisville Ridge.

Table 9. Genetic differentiation measures for population samples of orange roughy from the Louisville Ridge and the Box, based on all alleles. * significant test result; NA not applicable due to negative value.

Locus	Louisville		Box		Sum	
	F_{ST}/R_{ST}	*P*	F_{ST}/R_{ST}	*P*	F_{ST}/R_{ST}	*P*
F_{ST}						
OR2A	0.003	0.274	-0.004	NA	0.0003	0.380
OR40	0.004	0.234	-0.003	NA	0.008	0.020*
SUM	0.003	0.233	-0.004	NA	0.004	0.071
R_{ST}						
OR2A	0.034	0.071	0.009	0.226	0.020	0.085
OR40	0.029	0.093	0.010	0.166	0.010	0.188
SUM	0.032	0.050	0.009	0.194	0.015	0.078

11. Conclusions

1. Mitochondrial DNA haplotype markers showed no significant differences between samples from the Box, on the north Chatham Rise, and the Louisville Ridge. These markers had previously revealed differences among samples from the north Chatham Rise and Waitaki.
2. Two microsatellite loci, tested in New Zealand populations of orange roughy for the first time, revealed high levels of genetic variation.
3. There is a significant genetic difference among samples from the Box and the Louisville Ridge. One microsatellite locus, OR40, revealed a heterogeneity in allele frequencies among samples from the Box and the Louisville Ridge, but no heterogeneity within areas, indicating a genetic difference between the Louisville Ridge and the Box.

12. Publications

None

13. Data storage

Electronic copy of report held at NIWA Greta Point, tissue samples stored at -60^{0}C at NIWA Greta Point, data held in BIOSYS file at NIWA Greta Point.

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