Abstract.—Three types of genetic markers were used to determine genetic relations among four spawning populations of orange roughy off New Zealand. Eleven allozyme loci were tested in starch and cellulose acetate gels. Restriction fragment length polymorphisms were tested in two regions of the mitochondrial DNA amplified with the polymerase chain reaction. Random amplified polymorphic DNA (RAPD) products were generated with 10-base oligonucleotide primers and separated in agarose gels. There was a significant heterogeneity among all four populations, at 5 out of 11 allozyme loci, at 2 of 29 RAPD primer fragments, and in the frequency of mtDNA haplotypes. There was no significant difference between the two northern spawning populations for any marker, but there were significant differences between all other pairwise population comparisons with allozymes and RAPD's, indicating the presence of three genetic stocks. The mtDNA analysis revealed less genetic subdivision than did allozymes and RAPD's.

Manuscript accepted 14 May 1997.
Fishery Bulletin 95:800–811 (1997).

A comparison of three genetic methods used for stock discrimination of orange roughy, *Hoplostethus atlanticus*: allozymes, mitochondrial DNA, and random amplified polymorphic DNA

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The orange roughy, *Hoplostethus atlanticus*, is a deepwater species with wide distribution in the Atlantic, Indian, and South Pacific Oceans. Around New Zealand the species supports a fishery which peaked at 50,000 tons per annum in the mid 1980's but which has subsequently declined owing to quota restrictions. There are several geographically isolated spawning populations of orange roughy which are the major targets of fishing within the New Zealand Exclusive Economic Zone (EEZ).

A basic prerequisite of fisheries management is the identification of production units or stocks of a species; inadequate knowledge of stock structure may lead to over- or under-exploitation. Orange roughy occur at depths of about 1,000 m and therefore tag and release studies to estimate movements between areas are impracticable. There have been several other approaches to stock identification of orange roughy with differing results. Studies of parasite distribution (Lester et al., 1988), morphometric characters (Linkowski and Liwoch, 1986; Haddon and Willis, 1995), and trace element composition of otoliths (Edmonds et al., 1991) have demonstrated regional subdivisions in Australasian orange roughy. An allozyme study revealed a high level of genetic variation but only marginally significant differences between the fishing areas around New Zealand (Smith, 1986). Genetic evidence for discrete stocks off South Australia and eastern Australia based on an allozyme survey (Black and Dixon[1]) was not supported by a larger-scale study (Elliott and Ward, 1992). Restriction fragment length polymorphism (RFLP) analyses of mitochondrial (mt)DNA have indicated genetic subdivision of orange roughy around Australia (Smolenski et al., 1993) and New Zealand (Smith et al., 1996).

The development of the polymerase chain reaction (PCR), which amplifies DNA, enables genetic analyses to be carried out on small tissue samples and provides a range of methods for the population biolo-

[1] Black, M., and P. I. Dixon. 1989. Population structure of orange roughy (*Hoplostethus atlanticus*) in Australian waters. Internal Report, Centre for Marine Science, University of New South Wales, Kensington, Australia, 22 p.

gist without need for cloning and sequencing. PCR amplification of specific regions of mtDNA and digestion with restriction enzymes (PCR-RFLP) has been used as a fisheries tool for the differentiation of various fish species (Chow et al., 1993; Chow and Inoue, 1993) and for stock identification of albacore tuna (Chow and Ushiama, 1995), anchovies (Bembo et al., 1995), and salmonids (Cronin et al., 1993; Hall and Nawrocki, 1995; O'Connell et al., 1995; Hansen and Loeschcke, 1996). Mitochondrial DNA is maternally inherited and has a higher evolutionary rate relative to protein coding loci (Brown, 1983) and consequently has become a useful stock discrimination tool.

Random amplified polymorphic DNA (RAPD) uses PCR to amplify fragments of DNA with primers with random nucleotide sequences (Welsh and McClelland, 1990; Williams et al., 1990). Most fisheries applications of RAPD's have been at the species level (Dinesh et al., 1993; Bardakci and Skibinksi, 1994; Takagi and Taniguichi, 1995), although Macaranas et al. (1995) used RAPD's to distinguish populations of the freshwater red claw crayfish, *Cherax quadricarinatus,* in northern Australia, and a population specific RAPD marker was found in the marine shrimp *Penaeus vannamei* (Garcia et al., 1996).

In this paper we used three methods (allozymes, mtDNA, and RAPD's) to determine the genetic relations among orange roughy collected from four spawning sites off the east and south coasts of New Zealand.

Materials and methods

Tissue samples were collected on the RV *Tangaroa* from four spawning sites off the east and south coasts of New Zealand (Fig. 1). These sites were chosen because they are isolated by distances beyond the likely limit of larval drift (Zeldis et al., 1994). Each site supports significant fisheries, although the Waitaki fishery is relatively small and has declined quickly since development in the early 1990's (Annala and Sullivan[2]). Heart, liver, and muscle tissues were dis-

[2] Annala, J. H., and K. J. Sullivan. 1996. Report from the fishery assessment plenary, April–May 1996: stock assessments and yield estimates. Unpubl. Rep., Ministry of Fisheries, Greta Point Library, Wellington, New Zealand.

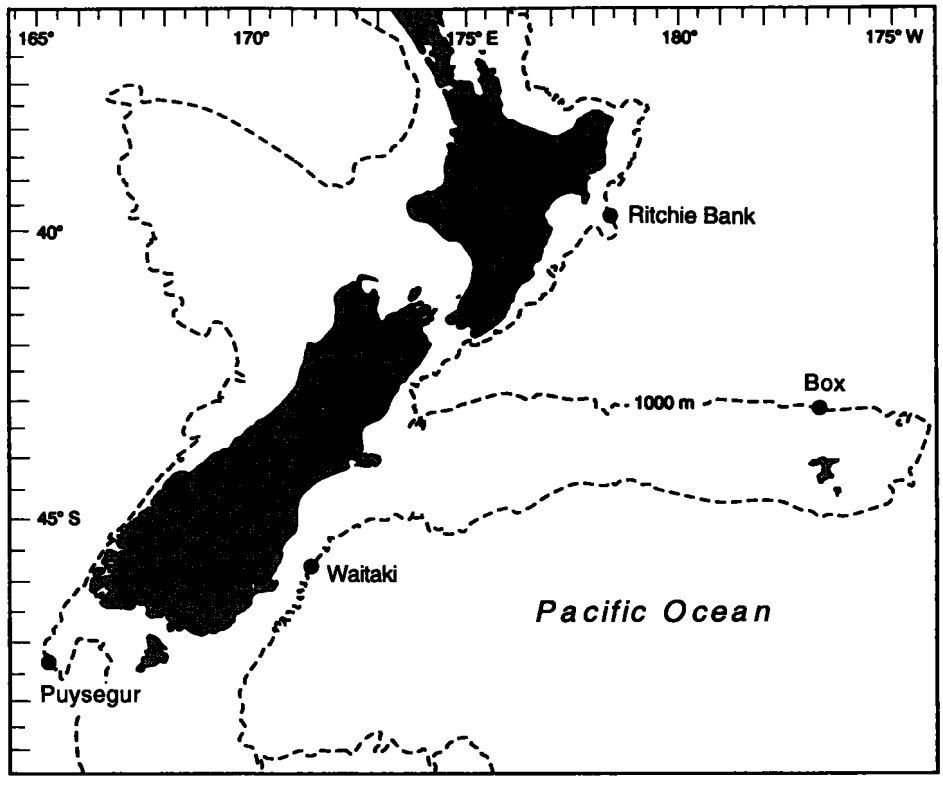

Figure 1

Location of orange roughy spawning sites around New Zealand sampled for genetic analyses. The dotted line represents the 1,000-m isobath.

sected from 100 specimens at three sites and from 50 specimens at Waitaki (Fig. 1). Tissue samples were frozen in liquid nitrogen at sea and stored at –70°C in the laboratory.

Allozyme electrophoresis

Eight enzyme systems were tested in heart, liver, and muscle tissues of orange roughy with cellulose acetate and starch gel electrophoresis following the methods in Smith (1986), except that BDH (British Drug House Chemicals Ltd, Poole, England) starch was substituted for Electrostarch (Electrostarch Company, USA).

DNA extraction

DNA was extracted from liver tissue of 50 orange roughy from each site. For each sample, 0.5 g of tissue was homogenized with 750 µL 4M guanidinium isothiocyanate in 8M urea and 2% sodium dodecyl sulfate (SDS) (Turner et al., 1989). DNA was extracted by mixing with an equal volume of phenol chloroform and centrifugation at 13,000 rpm for 5 min. The phenol-chloroform extraction was repeated and the aqueous fraction mixed with an equal volume of chloroform-isoamyl alcohol (24:1). Following centrifugation at 13,000 rpm, the aqueous fraction was mixed with two volumes of ethanol and the DNA allowed to precipitate at –20°C overnight. The DNA pellet was washed in 70% ethanol, air dried, and resuspended in 40 µL of sterile deionised water.

mtDNA amplification and restriction enzyme digestion

Three primer pairs were used to amplify the mtDNA. Amplification reactions were performed in 50-µL volumes in a Perkin Elmer Cetus DNA thermocycler: protocols followed those of Palumbi et al. (1991) and Cronin et al. (1993).The nucleotide sequences of the primers were the following:

D-loop	5'-ATAGTGGGGTATCTAATCCCA-3'
	5'-RCRCCCAAAGCTRRRRTTCTA-3'
	(Palumbi et al., 1991);
cytochrome *b*	5'-CCCTCAGAATGATA-TTTGTCCTCA-3'
	5'-TGACCTGAARAACCA-YCGTTG-3'
	(Palumbi et al.,1991); and
ND 5/6	5'-AATAGTTTATCCA-GTTGGTCTTAG-3'
	5'-TTACAACGATGGTTTTTCA-TAGTCA-3' (Cronin et al., 1993)

Twelve restriction endonucleases recognizing 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. Eleven restriction endonucleases recognizing 4-base sites (*Alu* I, *Bfa* I, *Cfo* I, *Hpa* II, *Msp* I, *Nar* I, *Rsa* I, *Sal* I, *Sau* 3A, *Taq* I, and *Tru* I) were used to digest the cytochrome *b* primer amplification products. The ND 5/6 primers produced between 1 and 3 amplification products in different specimens, therefore no restriction digests were undertaken with the PCR products.

For each primer pair and restriction enzyme, 24 fish were tested, 6 from each area. The restriction enzymes that showed polymorphisms were used to test 50 fish from each site. The amplified and digested DNA products were separated in 1.4% agarose gels and detected with ethidium bromide under a UV light (312 nm).

RAPD amplification and separation

Six individuals from each sample site were amplified with 24 RAPD primers. Each sample was amplified separately with a 10-base oligonucleotide primer from Operon (OperonTechnologies, Alameda, CA). These primers were randomly selected from Operon series A, D, E, and H primers, but all have a G+C content of 60–70%. Amplification reactions were performed in 50-µL volumes in a Perkin Elmer Cetus DNA thermocycler. Serial dilutions of DNA samples were tested initially to determine optimum DNA concentration for amplification (Fig. 2). The DNA concentration in each sample was estimated fluorometrically and appropriate volumes were used for amplification. Each reaction contained approximately 50 ng DNA in 10 mM Tris HCl (pH8.3), 30 ng single 10-base primer, 50 mM KCl, 2 mM $MgCl_2$, 100 mM each of *d*ATP, *d*CTP, *d*GTP, and *d*TTP, and 1 unit Taq DNA polymerase in Perkin Elmer PCR buffer. The reaction was overlaid with mineral oil and amplified. The thermocycler was programmed for 40 cycles of 1-min duration at 94°C, 1 min at 36°C, and 2 min at 72°C. Amplification products were separated in 1.4% agarose gels and detected with ethidium bromide staining under a UV light (312 nm). A DNA size-ladder was included in each gel. Control samples were amplified without a DNA template. Those primers that yielded variable fragment patterns were retested in the same fish. Primers producing repeatable fragment patterns in the initial six fish from each site were tested in 50 fish from each site. Polymorphisms were scored by the presence or absence of an amplification product at specific positions in the gel.

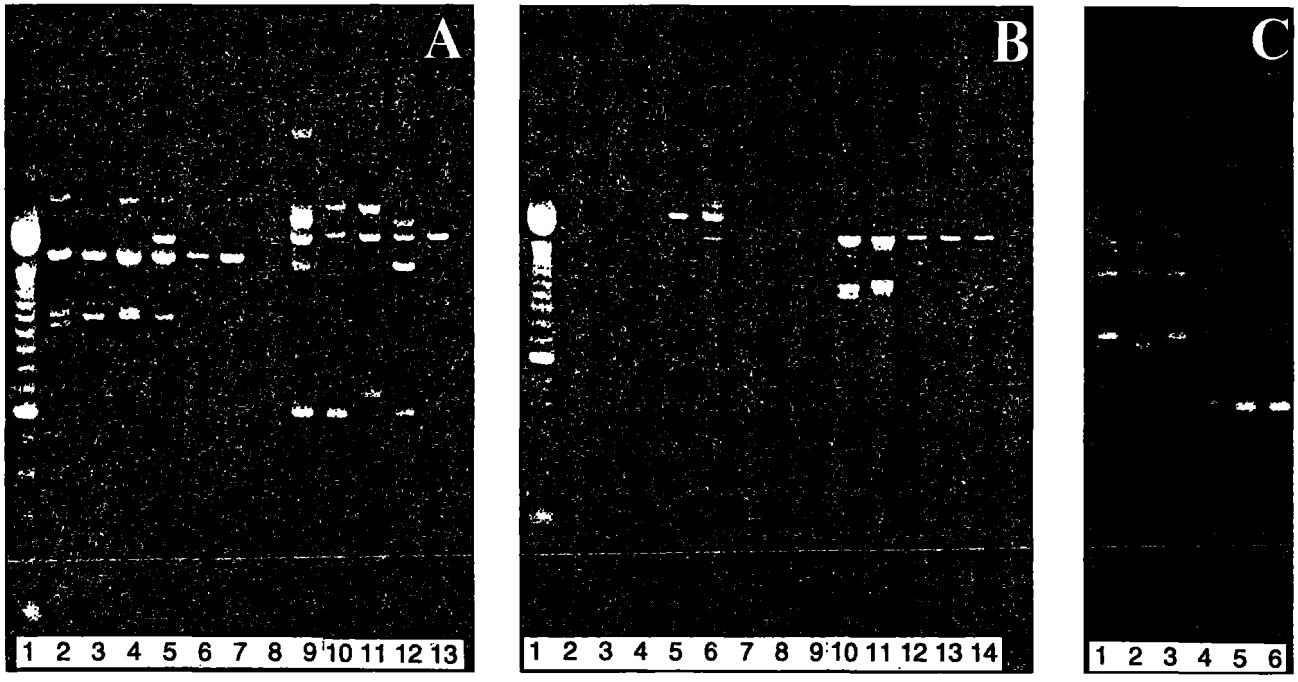

Figure 2

(**A**) Random amplified polymorphic DNA (RAPD) profiles in orange roughy generated with the primers A16 and E19. Lane 1 contains a DNA size-ladder (2,072–100 bp), lanes 2–7 represent orange roughy amplifed with primer A16, lane 8 contains no DNA template, and lanes 9–13 represent orange roughy amplifed with primer E19. Each amplified sample of orange roughy contained approximately 50 ng of DNA. (**B**) RAPD profiles in orange roughy generated with the primers A16 and E19 at different concentrations of DNA template. Lane 1 contains a DNA size-ladder (2,072–100 bp), lane 2 no DNA, lanes 3 and 4 contain 12.5 ng DNA amplified with E19, lanes 5 and 6 contain 50 ng DNA amplified with E19, and lanes 7 and 8 contain 200 ng DNA amplified with E19, lane 9 no DNA, lanes 10 and 11 contain 50 ng DNA amplified with A16, and lanes 12–14 contain 200 ng DNA amplified with A16. (**C**) RAPD profiles in orange roughy generated with the primer A14, lanes 1–3 contain 50 ng DNA, and lanes 4–6 represent the same samples at a concentration of 200 ng DNA.

Statistical analyses

Allozyme genotypes Genotypic frequencies were tested for Hardy-Weinberg equilibrium; weakly polymorphic loci (frequency of most common allele >0.95) were excluded. Rare heterozygotes were pooled with their nearest electrophoretic neighbor to reduce the number of cells with less than five observations. Allele frequencies were tested for heterogeneity among populations with contingency χ^2 tests with the BIOSYS software program (Swofford and Selander, 1981). To test for geographic structure, contingency χ^2 tests were undertaken on all pairwise combinations of populations. Probability levels were modified by the Bonferroni procedure for multiple tests according to Rice (1989).

The proportion of allozyme variation due to differentiation among populations was estimated with Nei's gene-diversity statistic G_{ST} (Nei, 1973), which is a multiallele estimator of Wright's F_{ST} statistics (Wright, 1951). Gene diversity is equal to

$$(H_T - H_S)/H_T,$$

where H_T = the total genetic diversity of all populations; and
H_S = the mean genetic diversity per population, calculated from the average expected heterozygosities.

Sampling error will produce differences in allele frequencies, even when samples are drawn from the same population, therefore a randomization test was used to test for differences due to sampling error (Elliott and Ward, 1992). One thousand randomizations were used, and the probability was estimated from the number of randomizations that were equal to or greater than the observed G_{ST}.

Gene diversity, G_{ST}, allows an estimation to be made of the number of migrants exchanged between populations per generation from the relation

$$N_e m = (1/G_{ST} - 1)/4,$$

where N_e = the effective population size; and
m = the rate of gene flow per generation.

It is assumed that m<<1 and that population differentiation is due to genetic drift and migration with no selection. Gene diversity was corrected to a "true" estimate by subtracting the G_{STnull} due to sampling error, derived from a randomization test (Elliott and Ward, 1992).

mtDNA Heterogeneity in haplotype frequencies in the total data was tested by the χ^2 randomization test described by Roff and Bentzen (1989) with 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 1,000 random rearrangements of the data. In addition the χ^2 randomization test was applied to pairwise comparisons of all populations to test for geographic structure. Probabilities were estimated from the number of randomizations that were equal to or greater than the observed χ^2 value. The proportion of haplotype variation due to differentiation between populations was estimated by G_{ST} from the haplotype frequencies, as for allozymes. The number of migrants exchanged per generation was estimated from the relation

$$N_e m_f = (1/G_{ST} - 1)/2,$$

where m_f= female migration, modified to account for the maternal inheritance of mtDNA.

RAPD Standard genetic calculations are not immediately applicable to RAPD data because the fragments are dominant: individuals carrying two copies of an allele cannot be distinguished from individuals carrying one copy of the allele. Black (1995) has provided a set of programs for analyzing RAPD population data but points out that a number of assumptions have to be made. First, the observed fragments are dominant alleles and the absent fragments are recessive alleles. Second, the genotypes are in Hardy-Weinberg equilibrium and each observed polymorphism is biallelic: all the absent observations are produced by the same recessive allele and all the present observations are produced by a single dominant allele with or without the recessive allele. Each primer was scored for the presence or absence of fragments in the gel. Each fragment, regardless of primer, was treated as an independent locus. In most RAPD studies, fragments have been found that vary in staining intensity; we scored only fragments that were intensely stained, following Black (1993).

Random amplified polymorphic DNA allele frequencies were calculated from the presence or absence observations with the RAPDBIOS software program (Black, 1995) and then used in the BIOSYS software program (Swofford and Selander, 1981) for calculation of heterogeneity in allele frequencies as for allozyme data. The gene-diversity statistic G_{ST} (=F_{ST}) was calculated with the RAPDFST software program (Black, 1995); probabilities were calculated according to Workman and Niswander (1970). An estimation of the number of migrants exchanged per generation, $N_e m$, was estimated as for the allozyme data.

Results

Allozymes

Eleven enzyme loci were resolved in the four populations and allele frequencies are given in Appendix Table 1. Eight loci were sufficiently polymorphic (P<0.95) for Hardy-Weinberg tests. One out of a possible 32 tests (8 loci × 4 populations) showed a significant departure from Hardy-Weinberg equilibrium when a Bonferroni modified probability level was applied (*Idh-1** Puysegur, χ^2=13.99, 1 df, P<0.001).

The polymorphic loci were tested with a contingency χ^2 test. There was a significant heterogeneity among all four populations at 5 loci, *Est-1**, *Gpi-2**, *Idh-1**, *Idh-2**, and *Ldh-1**, with a Bonferroni-modi-

Table 1

Results of comparisons of allele frequencies at eleven loci and mtDNA haplotypes in four populations of orange roughy. df = degrees of freedom; P = probability value; and G_{ST} = gene diversity. * = significant at the 5% level with a Bonferroni-modified P for multiple tests.

Locus	χ^2	df	P	G_{ST}	P
*Cck-1**	7.93	6	0.243	0.006	0.277
*Est-1**	63.09	12	<0.001*	0.030	<0.001*
*Gpi-1**	4.70	9	0.860	0.002	0.889
*Gpi-2**	25.09	6	<0.001*	0.021	0.002*
*Idh-1**	26.91	9	0.001*	0.026	<0.001*
*Idh-2**	46.08	9	<0.001*	0.065	<0.001*
*Ldh-1**	19.02	3	0.003*	0.025	0.004*
*Ldh-2**	12.09	6	0.061	0.008	0.153
*Mdh-1**	11.20	6	0.082	0.012	0.066
*Mpi-1**	7.56	9	0.581	0.003	0.653
*Pgm-1**	2.58	6	0.860	0.001	0.839
all loci	226.2	81	<0.001	0.020	<0.001
mtDNA haplotypes	45.51		0.001	0.057	0.001

Table 2

Heterogeneity χ^2 pairwise comparisons for allozyme loci, mtDNA haplotypes, and random amplified polymorphic DNA (RAPD) fragments, among four populations of orange roughy. For the allozyme and RAPD data only those loci and fragments that were significant applying a Bonferroni-modified probability level are given.

Pair	Allozyme loci and probabilities	mtDNA haplotype probablities	RAPD primer fragments and probabilities	
Ritchie and Box	NS NS	NS		
Ritchie and Waitaki	*Est-1** $P<0.001$	0.001	E19-3	$P<0.001$
Ritchie and Puysegur	*Gpi-2** $P<0.001$, *Idh-2** $P<0.001$	NS	A16-1	$P<0.001$
Box and Waitaki	*Est-1** $P<0.001$, *Idh-1** $P=0.001$ *Idh-2** $P<0.001$	NS	E19-3	$P<0.001$
Box and Puysegur	*Idh-2** $P<0.001$	NS	A16-1	$P<0.001$
Waitaki and Puysegur	*Est-1** $P<0.001$	0.003	E19-3	$P<0.001$

fied P for 11 loci (Table 1). To test for geographic structure, additional χ^2 tests were carried out on all pairwise combinations of populations. There was a significant heterogeneity for at least one locus between all population pairs, except Ritchie Bank and Box (Table 2).

The heterogeneity in the total data was confirmed by the gene diversity analysis (Table 1). When a Bonferroni-modified P is applied, the 5 loci show a G_{ST} significantly greater than that due to sampling error. Over all eleven loci G_{ST} was 0.020 (Table 1), indicating that around 2% of the observed genetic variation was due to differences among populations. From this estimate of G_{ST}, and by subtracting the G_{STnull}, the minimum number of effective migrants per generation (N_em) was 13.2 (Table 3). Individual pairs *of* N_em varied from 15.7 (Box and Waitaki) to 124 (Ritchie and Box).

Table 3

The estimated number of migrants exchanged per generation (N_em) for allozyme, mtDNA, and random amplified polymorphic DNA (RAPD) data sets of orange roughy.

Population	Allozyme	mtDNA	RAPD
Ritchie and Box	124.0	277.8	75.0
Ritchie and Waitaki	14.7	7.2	7.7
Ritchie and Puysegur	19.4	35.7	18.0
Box and Waitaki	15.7	18.3	7.8
Box and Puysegur	25.3	36.5	16.0
Waitaki and Puysegur	75.5	9.6	6.5
Total	13.2	9.8	7.0

mtDNA

The estimated size of the PCR amplified D-loop was 1,500 base pairs and that of the cytochrome *b* was 500 bp. Four restriction enzymes, *BstU* I, *Cfo* I, *Msp* I, and *Nla* III, produced two or more fragment patterns with the D-loop primers (e.g. Fig. 3) and were tested in all fish. For each area, a few fish samples failed to produce an amplification product; the same fish samples also failed to produce an amplification product with the RAPD primers. Four restriction enzymes, *Alu* I, *Bfa* I, *Rsa* I, and *Taq* I, showed variation in the first 24 fish tested with the cytochrome *b* primers, but the variation was limited to a single individual with each restriction enzyme. No further amplifications were undertaken with this set of primers. The numbers of haplotypes observed at each site are shown in Appendix Table 2. There is a significant heterogeneity in the total data ($P=0.001$), with only 1 out of 1,000 randomizations exceeding the original χ^2 value (Table 1). In pairwise comparisons of the four spawning populations (Table 2), significant differences were found between Ritchie Bank and Waitaki ($P<0.001$) and between Waitaki and Puysegur ($P=0.001$), but not in the other pairwise comparisons.

Gene diversity was estimated to be 0.057 (Table 1), which is significantly greater than that due to sampling error, and indicates that around 6% of the observed genetic variation is due to differences among populations. From this estimate of G_{ST}, and by subtracting the G_{STnull}, the minimum number of female migrants per generation (N_em_f) among the four populations was estimated to be 9.8 (Table 3). The pairwise values varied from 7.2 (Ritchie and Waitaki) to 277.8 (Ritchie and Box).

RAPD

Seven primers tested in 24 orange roughy produced clear DNA fragments and the same profiles in repeat tests. The primers (and their sequences 5' to 3')

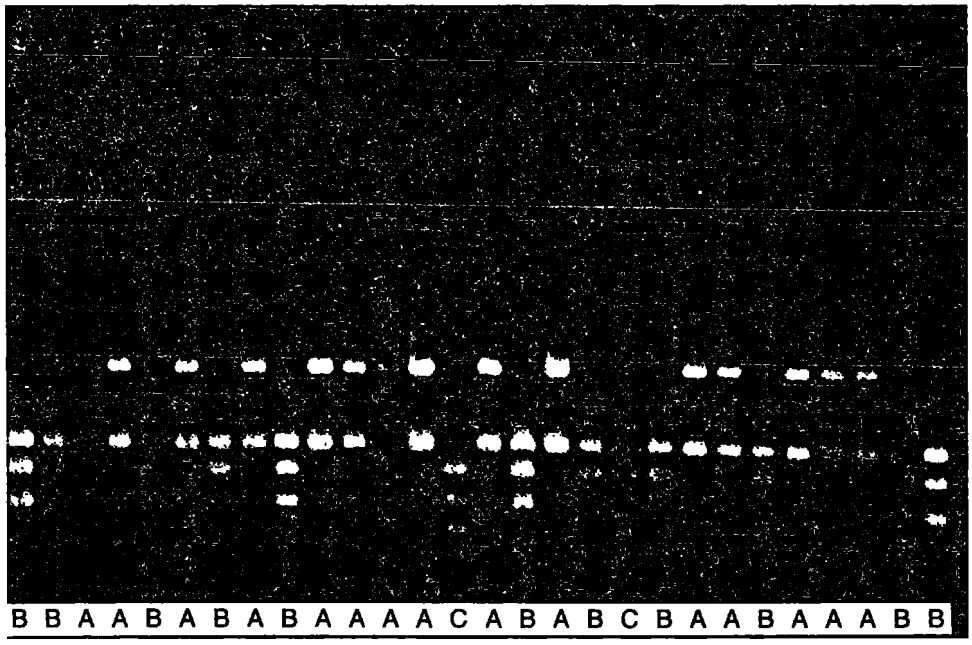

Figure 3

Photograph of *Cfo 1* haplotypes in the D-loop region of mtDNA amplified from 28 orange roughy. Haplotype A = 2 fragments, B = 3 fragments, and C = 4 fragments.

were A14 (TCTGTGCTGG), A15 (TTCCGAACCC), A16 (AGCCAGCGAA), A17 (GACCGCTTGT), D15 (CATCCGTGCT), E19 (ACGGCGTATG), and H17 (CACTCTCCTC). The number of scored fragments varied from 1 to 6 per primer, and the size of the fragments from 0.6 to 2.8 kb. Fragments that could be scored were numbered in decreasing order of electrophoretic mobility (e.g. primer A14 fragment 1 = A14-1); each individual fish was scored for the presence or absence of each fragment. Repeat tests on some individuals did not produce repeatable patterns for some weakly staining fragments, therefore presence or absence of each fragment was not scored for these fragments. Omitting the DNA template from the PCR reaction (i.e. negative control) failed to produce fragments. The amount of DNA in the initial extractions varied tenfold between samples. Excess DNA, 250 ng, produced different fragment patterns with some primers (Fig. 2), therefore all amplifications were optimized to contain a 50-ng template of DNA.

The estimated allele frequencies are given in Appendix Table 3. Two out of 29 primer fragments (A16-1, E19-3) revealed a significant heterogeneity among populations when a heterogeneity χ^2 test with a modified probability for multiple tests was applied (Table 4). Pairwise comparisons showed significant differences between all pairs of populations except Ritchie Bank and Box at these two primer fragments (Table 2). The heterogeneity in the total data set was confirmed by the G_{ST} tests (Table 4). The effective number of migrants per generation was estimated to be 7.0 from the overall G_{ST}, minus G_{STnull} due to sampling error (Table 3), as described for allozymes. Pairwise values varied from 6.5 (Waitaki and Puysegur) to 75 (Ritchie and Box).

Discussion

There was significant heterogeneity in the allozyme data set at five loci (Table 1) which indicated that the population samples had not been taken from a single panmictic stock. Pairwise comparisons showed that there were significant differences between all pairs of spawning populations except the two northern populations at Ritchie Bank and Box (Fig. 1). The RAPD data also showed a significant heterogeneity that indicated that the population samples had been taken from more than one genetic unit stock. There were no area-specific RAPD fragments in orange roughy, as have been reported in marine prawns (Garcia et al., 1996) and freshwater crayfish (Macaranas et al., 1995), but there were differences in

frequencies of two primer fragments (A16-1, E19-3, Table 4). As with the allozyme data, there were differences between all pairwise comparisons, with the exception of those samples taken at Ritchie Bank and Box (Table 2).

The mtDNA data also showed a significant heterogeneity in the total data set but demonstrated less genetic differentiation than the allozyme and RAPD data sets, with only two pairwise comparisons showing a significant difference (Table 2). However all three methods, which have measured different parts of the genome, gave similar results of low genetic exchange among the four populations (Table 3). None of the estimates of N_em are true estimates because our data sets are biased in favor of polymorphic markers, which will tend to inflate the G_{ST} estimate; such estimates of N_em (Table 2) can be used to compare only relative levels of geneflow between areas (Ferguson, 1994). In this respect there is 8–10 times as much gene flow between Ritchie Bank and Box than between these sites and Waitaki, when measured with allozymes and RAPD's, and 15–38 times as much with mtDNA (Table 3). Significant genetic differences between spawning groups provides evidence of genetic isolation, and thus the data reveal three genetic groups: 1) Puysegur, 2) Waitaki, and 3) Ritchie Bank and Box (Table 2).

There are problems with RAPD analyses that may preclude them from use as stock markers for orange roughy. Because RAPD markers are dominant, a number of assumptions have to be made to analyze the data (Lynch and Milligan, 1994). Some of these assumptions, in particular that fragments with the same electrophoretic mobility are genetically identical and that absent fragments represent the same DNA fragment, may not be valid. Fragments that exhibited weak staining activity were not scored, so that there is a subjective element when scoring RAPD gels.

In the absence of breeding studies, the allelic nature of presence or absence of RAPD fragments may be suspect. Garcia and Benzie (1995) reported an extra RAPD fragment in prawn larvae that was absent in adults, although they found Mendelian inheritance of other RAPD markers. Unlike the other two genetic methods, there are no internal checks that can be used to fit RAPD phenotypes to a genetic model: with allozymes there is an expected gel phenotype for each enzyme and all alleles are equally expressed; with mtDNA the size of the restricted fragments should add up to the size of the undigested fragment.

Some primers produced weak fragments that were not repeatable in reamplifications. These weak fragments may be produced by excessive PCR cycles; Bell and DeMarini (1991) have shown that by increasing the number of PCR cycles above 30, nonspecific DNA products can be obtained. However, in our preliminary amplifications in extracting DNA from frozen tissue samples, less than 40 cycles produced faint fragment patterns for most primers; thus 40 cycles were used as a standard. The RAPD technique has been shown to be very sensitive to changes in concentration of primer, concentration of template, annealing temperature, and the concentration of magnesium ions, all of which can affect the number and intensity of bands (Devos and Gale, 1992; Ellsworth et al., 1993; Patwary et al., 1993; Penner et al., 1993). We sought to avoid these problems by standardizing DNA quantities prior to amplification, performing all amplifications on the same thermocycler, and using the same batch of chemicals. Tissue samples from the four spawning sites were collected and stored under similar conditions.

Table 4

Heterogeneity χ^2 tests and gene diversity (G_{ST}) for seven random amplified polymorphic DNA (RAPD) primers in four populations of orange roughy. df = degrees of freedom; P = probability value; G_{ST} = gene diversity. (* = significant at Bonferroni-modified P for multiple tests).

Primer and fragment	χ^2 (3 df)	P	G_{ST} (3 df)	P
A14-1	2.820	0.422	0.010	0.415
A14-2	3.306	0.347	0.012	0.341
A14-3	9.089	0.028	0.032	0.031
A15-1	1.543	0.672	0.006	0.671
A15-2	8.189	0.042	0.029	0.044
A15-3	3.593	0.309	0.013	0.299
A15-4	2.138	0.544	0.008	0.540
A16-1	16.921	<0.001*	0.079	<0.001*
A16-2	1.473	0.688	0.005	0.685
A16-3	6.874	0.076	0.024	0.087
A16-4	5.072	0.167	0.019	0.156
A16-5	0.513	0.916	0.002	0.915
A17-1	2.138	0.544	0.008	0.540
A17-3	6.715	0.082	0.025	0.073
D15-1	5.436	0.143	0.018	0.178
D15-2	2.114	0.549	0.008	0.513
D15-3	3.862	0.277	0.014	0.280
D15-4	1.678	0.642	0.007	0.615
E19-1	8.080	0.044	0.300	0.043
E19-2	9.283	0.026	0.032	0.033
E19-3	23.079	<0.001*	0.082	<0.001*
E19-4	6.558	0.087	0.026	0.071
E19-5	2.680	0.444	0.010	0.423
E19-6	0.823	0.844	0.002	0.887
H17-1	3.824	0.281	0.014	0.268
H17-2	3.119	0.374	0.012	0.343
H17-3	0.776	0.855	0.003	0.863
H17-4	2.155	0.541	0.007	0.604
H17-5	0.500	0.919	0.002	0.913
Total (87 df)	164.66	<0.001	0.019	<0.001

Given the technical problems with RAPD's, we would recommend them only when other genetic methods have failed to reveal polymorphisms. Techniques such as PCR-RFLP of mtDNA, or allozymes, yielded fewer polymorphisms per unit of laboratory time than did RAPD's but still produced sufficient polymorphisms to detect population structure in orange roughy. Our allozyme data set indicated a higher level of genetic subdivision than that found with mtDNA in orange roughy. This result is surprising in view of the relatively higher rate of evolution of mtDNA (Brown, 1983), and it is possible that other regions of the mitochondrial genome, or use of additional restriction enzymes, might reveal more genetic variation. Several studies of marine organisms have detected greater genetic subdivision with mtDNA than with allozyme markers (e.g. Reeb and Avise, 1990), although there are examples of the reverse in the fisheries literature (Grewe et al., 1994; Ward et al., 1994). It is possible that the allozyme markers are under selection (Koehn et al., 1980) and are responding to short-term population events rather than to historical events due to reproductive isolation.

Acknowledgments

We are grateful to Malcolm Clark and Di Tracey, for collection of orange roughy tissues samples, and to John Patton and two anonymous referees for constructive comments on the manuscript. This research was supported by funds from The Exploratory Fishing Company (ORH3B) Limited and the New Zealand Ministry of Fisheries, project number FBOR01.

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Appendix

Appendix Table 1

Allele frequencies for 11 allozyme loci tested in four populations of orange roughy.

Locus	Allele (*n*=no. of fish)	Ritchie	Box	Waitaki	Puysegur
*Cck-1**	1	0.494	0.500	0.596	0.551
	2	0.489	0.500	0.404	0.444
	3	0.017	0.000	0.000	0.005
	n	90	81	47	99
*Est-1**	1	0.051	0.160	0.093	0.152
	2	0.222	0.229	0.372	0.250
	3	0.709	0.606	0.430	0.585
	4	0.006	0.005	0.006	0.013
	5	0.013	0.000	0.000	0.000
	n	79	94	43	99
*Gpi-1**	1	0.515	0.551	0.553	0.517
	2	0.232	0.253	0.245	0.265
	3	0.253	0.191	0.191	0.214
	4	0.000	0.006	0.001	0.004
	n	99	89	47	99
*Gpi-2**	1	0.015	0.033	0.042	0.058
	2	0.970	0.944	0.948	0.860
	3	0.015	0.022	0.010	0.081
	n	99	90	48	99
*Idh-1**	1	0.030	0.011	0.106	0.083
	2	0.970	0.979	0.883	0.897
	3	0.000	0.005	0.011	0.021
	4	0.000	0.005	0.000	0.000
	n	99	94	47	99
*Idh-2**	1	0.417	0.442	0.180	0.196
	2	0.583	0.548	0.809	0.793
	3	0.000	0.005	0.011	0.011
	4	0.000	0.005	0.000	0.000
	n	96	94	47	94
*Ldh-1**	1	1.000	0.983	0.979	0.931
	2	0.000	0.017	0.021	0.069
	n	96	90	47	99
*Ldh-2**	1	0.000	0.000	0.011	0.000
	2	1.000	0.980	0.989	0.980
	3	0.000	0.020	0.000	0.020
	n	96	99	47	99
*Mdh-1**	1	0.625	0.729	0.656	0.745
	2	0.375	0.271	0.344	0.250
	3	0.000	0.000	0.000	0.005
	n	96	94	48	98
*Mpi-1**	1	0.037	0.027	0.052	0.057
	2	0.957	0.968	0.948	0.943
	3	0.000	0.005	0.000	0.000
	4	0.005	0.000	0.000	0.000
	n	94	94	48	97
*Pgm-1**	1	0.116	0.144	0.117	0.126
	2	0.879	0.850	0.883	0.874
	3	0.005	0.006	0.000	0.000
	n	99	90	47	99

Appendix Table 2

Numbers of composite mtDNA D-loop haplotypes observed in four populations of orange roughy. The composite haplotypes are based on the restriction enzymes *BstU* I, *Cfo* I, *Msp* I, and *Nla* III.

Haplotype	Ritchie	Box	Waitaki	Puysegur
AABA	21	19	5	14
BBBA	11	18	21	7
ACBA	0	0	2	0
AAAA	2	0	0	0
AABB	1	1	1	3
AABC	1	0	0	0
AACA	1	0	0	0
ABBA	4	4	6	10
BABA	2	6	6	6

Appendix Table 3

Random amplified polymorphic DNA (RAPD) fragment frequencies, calculated by assuming a biallelic system in Hardy-Weinberg equilibrium, in four populations of orange roughy.

Locus	Allele (*n*=no. of fish)	Ritchie	Box	Waitaki	Puysegur
A14-1	1	0.00	0.021	0.00	0.021
	2	1.00	0.979	1.00	0.979
	n	44	48	42	40
A14-2	1	0.454	0.417	0.417	0.330
	2	0.546	0.583	0.583	0.670
	n	44	48	42	40
A14-3	1	0.306	0.188	0.167	0.394
	2	0.694	0.813	0.833	0.596
	n	44	48	42	40
A15-1	1	0.009	0.00	0.00	0.00
	2	0.991	1.00	1.00	1.00
	n	44	48	42	40
A15-2	1	0.806	0.792	0.708	0.638
	2	0.194	0.208	0.292	0.362
	n	44	48	42	40
A15-3	1	0.028	0.042	0.042	0.00
	2	0.972	0.958	0.958	1.00
	n	44	48	42	40
A15-4	1	0.009	0.021	0.00	0.00
	2	0.991	0.979	1.00	1.00
	n	44	48	42	40
A16-1	1	0.49	0.46	0.33	0.74
	2	0.51	0.54	0.67	0.26
	n	43	48	42	38
A16-2	1	0.019	0.00	0.00	0.021
	2	0.981	1.00	1.00	0.979
	n	43	48	42	38
A16-3	1	0.278	0.271	0.125	0.149
	2	0.722	0.729	0.875	0.851
	n	43	48	42	38
A16-4	1	0.694	0.708	0.583	0.564
	2	0.306	0.292	0.417	0.436
	n	43	48	42	38
A16-5	1	0.019	0.021	0.00	0.021
	2	0.981	0.979	1.00	0.979
	n	43	48	42	38
A17-1	1	0.009	0.021	0.00	0.00
	2	0.991	0.979	1.00	1.00
	n	43	48	42	38
A17-3	1	0.009	0.00	0.00	0.053
	2	0.991	1.00	1.00	0.947
	n	43	48	42	38
D15-1	1	0.111	0.188	0.042	0.074
	2	0.889	0.813	0.958	0.926
	n	44	48	42	40
D15-2	1	0.426	0.542	0.500	0.489
	2	0.574	0.458	0.500	0.500
	n	44	48	42	40
D15-3	1	0.629	0.708	0.583	0.745
	2	0.361	0.292	0.417	0.255
	n	44	48	42	40
D15-4	1	0.083	0.063	0.042	0.043
	2	0.907	0.938	0.958	0.957
	n	44	48	42	40
E19-1	1	0.269	0.292	0.142	0.330
	2	0.731	0.708	0.858	0.670
	n	43	48	42	38
E19-2	1	0.148	0.271	0.042	0.149
	2	0.852	0.729	0.958	0.851
	n	43	48	42	38
E19-3	1	0.639	0.646	0.125	0.521
	2	0.361	0.354	0.875	0.479
	n	43	48	42	38
E19-4	1	0.093	0.021	0.083	0.021
	2	0.898	0.979	0.917	0.979
	n	43	48	42	38
E19-5	1	0.056	0.042	0.00	0.021
	2	0.944	0.958	1.00	0.979
	n	43	48	42	38
E19-6	1	0.769	0.708	0.708	0.745
	2	0.231	0.292	0.292	0.255
	n	43	48	42	38
H17-1	1	0.046	0.00	0.00	0.021
	2	0.954	1.00	1.00	0.979
	n	44	48	42	38
H17-2	1	0.806	0.708	0.708	0.713
	2	0.194	0.292	0.292	0.287
	n	44	48	42	38
H17-3	1	0.769	0.792	0.708	0.745
	2	0.231	0.208	0.292	0.255
	n	44	48	42	38
H17-4	1	0.731	0.708	0.708	0.638
	2	0.269	0.292	0.292	0.362
	n	44	48	42	38
H17-5	1	0.019	0.021	0.042	0.021
	2	0.981	0.979	0.958	0.979
	n	44	48	42	38