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Title: GENETIC COMPARISON OF DESCHUTES RIVER STEELHEAD AND RAINBOW TROUT AT SELECTED ENZYME LOCI

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John D. McIntyre

An electrophoretic enzyme examination to compare the genetics of anadromous and non-anadromous rainbow trout (Salmo gairdneri) from the Deschutes River in central Oregon was conducted. Four enzymes; lactate dehydrogenase, malate dehydrogenase, esterase and tetrazolium oxidase were found to be polymorphic in most groups of trout studied and were also shown to be inherited in a predictable manner.

With the exception of trout sampled from Jordan Creek, no statistically significant genetic differences were shown among seven groups of steelhead and rainbow trout compared. Trout from Jordan Creek were isolated above several barrier waterfalls and were found to be genetically different from any other group of anadromous or non-anadromous trout studied.

Genetic Comparison of Deschutes River Steelhead and Rainbow Trout at Selected Enzyme Loci

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Mark William Chilcote

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Assistant Professor of Fisheries and Wildlife
in charge of major

Redacted for privacy

Head of Department of Fisheries and Wildlife

Redacted for privacy

Dean of Graduate School

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TABLE OF CONTENTS

LIST OF TABLES

GENETIC COMPARISON OF DESCHUTES RIVER STEELHEAD AND RAINBOW TROUT AT SELECTED ENZYME LOCI

I. INTRODUCTION

Anadromous (steelhead trout) and non-anadromous (rainbow trout) forms of the same species, Salmo gairdneri, coexist in Oregon's Deschutes River. They utilize common spawning grounds and interbreeding has been observed (personal communication, J. Fessler, Research Biologist, Oregon Department of Fish and Wildlife). In addition, there is evidence that significant numbers of steelhead smolts released from Round Butte Fish Hatchery remain and reproduce in the river (personal communication, J. Fessler).

There is some evidence that certain electrophoretically detectable enzyme genotypes are more common in migratory forms of rainbow trout. One population of trout known to migrate downstream and inhabit a freshwater lake was shown to have a higher proporation of lactate dehydrogenase BB genotypes than a non-migratory population from the same stream, isolated above a barrier waterfall (Northcote et al., 1970). In addition, Huzyk and Tsuyuki (1973) found that lactate dehydrogenase BB genotypes were more common in ocean-migratory or anadromous populations than in the non-anadromous populations.

A genetic difference between anadromous and non-anadromous trout populations may occur because of the action or interaction of three important factors: 1) certain genotypes of a polymorphic enzyme or enzymes may be correlated with anadromous or non-anadromous behavior; 2) the genotypes of certain polymorphic enzymes may be influenced by different selective forces in the ocean and in freshwater and 3) steelhead and resident rainbow trout may be distinct races.

The objective of this study was to describe the genetic relationships between anadromous and non-anadromous forms of Deschutes River rainbow trout. Comparisons of the karyotypes (Wilmot, 1973) and melting properties of DNA (Gharrett, 1974) for the Deschutes River trout failed to discriminate between these two forms. Genotype frequencies as determined by protein electrophoresis were used in this study as a basis for comparison of these fish.

II. METHODS AND MATERIALS

Specimen Sampling

Fish were obtained from eight groups of Deschutes River trout. The sample locations are identified on Figure 1. For the purposes of discussion each group was assigned a short name which is underlined in the following description. In April 1973 two groups of adult steelhead were sampled at Pelton Dam; one of hatchery origin (hatchery steelhead) and one from the native stock (wild steelhead). Steelhead smolts produced at Round Butte Dam Fish Hatchery in 1971 were marked by removing certain fins prior to release. A portion of these marked fish remained in the river and never migrated downstream to the ocean. In September 1973, a sample of these trout (residual steelhead) distinguishable by missing fins, were taken immediately below Pelton Dam.

A sample of adult resident trout (mainstem rainbow) were collected from the Deschutes River near Maupin in April 1972. In April 1974 a sample of residual juvenile trout from Bakeoven Creek (Bakeoven juveniles) was obtained. Adult resident trout were obtained in April 1972 and downstream migrant trout were captured in May 1973 from Buck Hollow Creek. The names assigned these two groups were Buck Hollow rainbow and Buck Hollow smolts, respectively. Finally a sample of juvenile trout from Jordan Creek (Jordan juveniles) was

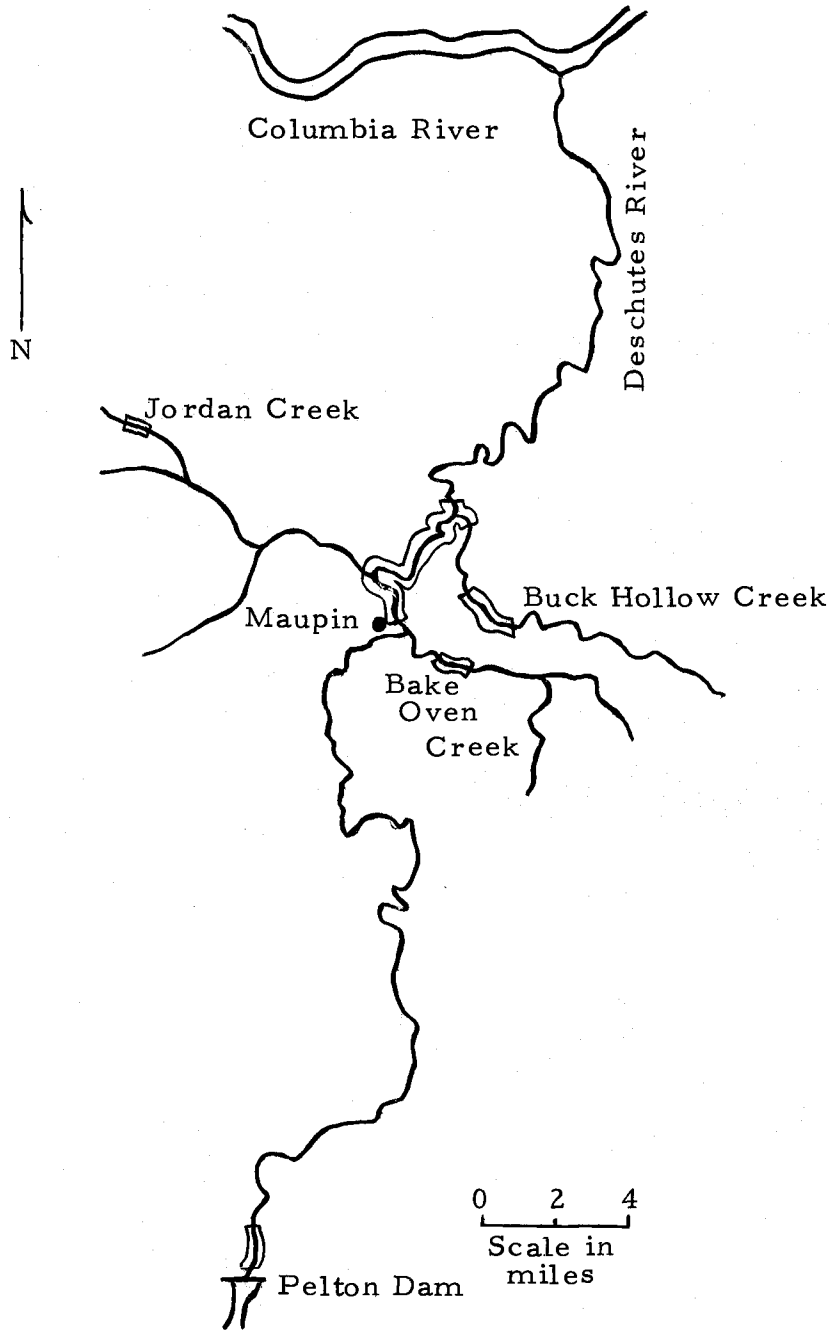


Figure 1. Map of lower Deschutes River showing fish collection sites.

obtained in April 1974. Fish from Jordan Creek were isolated from all other groups by several impassable waterfalls. Most fish were obtained by electrofishing. Adult steelhead were obtained from an upstream migrant trap at Pelton Dam. Buck Hollow smolts were caught in a trap for downstream migrants at the mouth of Buck Hollow Creek.

Principles of Electrophoresis

Certain proteins found in trout are known to exhibit variable biochemical characteristics. The variability of a protein from individual to individual can be detected by measuring the relative migration rate of these protein variants through an electrical field. This technique, referred to as electrophoresis, has opened new possibilities for assessing the genetic variation within and between trout populations.

The genetic interpretation of electrophoretic data is based on the assumption that a specific gene codes for specific protein sub-unit and variations of these genes will result in structural variations in the corresponding protein. However, this assumption must be demonstrated as valid if genetic interpretations are to be discussed. To confirm that electrophoretically determined enzyme variants were expressions of polymorphic loci several pairs of adults were mated and the progeny from each cross were reared. When the progeny were

one year old, the frequency of enzyme variants found in each group was compared to the frequencies expected from the parent types.

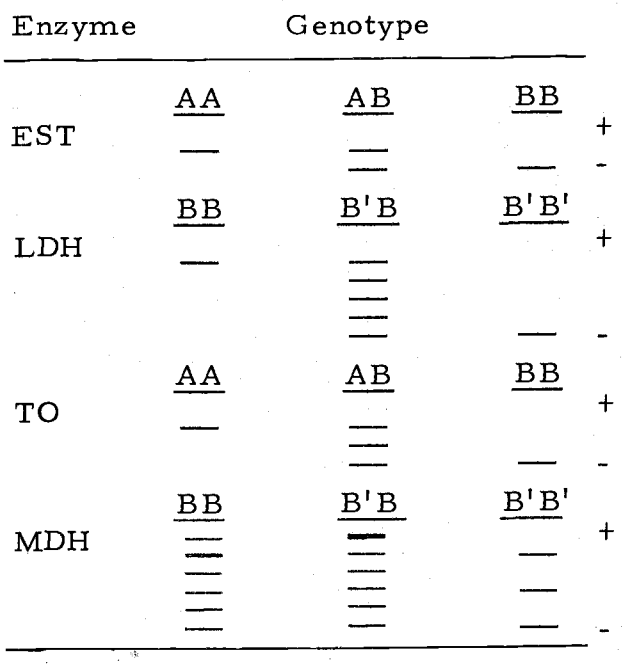
Electrophoresis

Paper wicks soaked with water suspended tissue extracts were imbedded into a horizontal starch gel. An electric current was applied to the gel for 3 to 6 hours. The gel then was put into an enzyme specific staining solution. This procedure is similar to what has been described by Kristjannson (1963) and Brewer (1970). Two buffer systems were used; a tris-citric acid, lithium hydroxide-boric acid buffer previously described by Ridgeway et al. (1970) and sodium phosphate buffer described by Wolf et al. (1970). The specific procedures for mixing the buffer solutions as well as 16 different enzyme stains are given in Appendix I.

III. RESULTS

Sixteen enzyme systems were screened for resolution and the presence or absence of variants (Table 1). The decision to use lactate dehydrogenase (LDH), malate dehydrogenase (MDH), tetrazolium oxidase (TO), and esterase (EST) for testing the genetic distinctiveness of Deschutes River rainbow and steelhead resulted from this initial screening process. Variants were also found for transferrin and isocitrate dehydrogenase systems. However, a full examination of the two latter systems was not completed because consistent resolution was not achieved (Table 1).

The electrophoretic patterns observed and the genetic interpretation for each system utilized were:



In all cases migration direction of enzyme was anodal.

Table 1. Results of initial screening of Deschutes River rainbow trout for 16 enzyme systems.

Enzyme	Tissue	Buffer System	Resolution	Variants Present
Lactate dehydrogenase (LDH)	liver	I	+	yes
Malate dehydrogenase (MDH)	white muscle	II	+	yes
Tetrazolium oxidase (TO)	liver	I	+	yes
Esterase (EST)	liver	I	+	yes
Isocitrate dehydrogenase (IDH)	liver	II	0	yes
General protein (GP)	blood serum	I	0	yes
Alpha glycerophosphate dehydrogenase (AGPD)	white muscle	II	0	none observed
Sorbital dehydrogenase (SDH)	liver	I	0	none observed
Phosphoglucomutase (PGM)	white muscle	I	+	none observed
Succinic dehydrogenase (SucDH)	liver	I	-	none observed
Aspartate aminotransferase (AAT)	white muscle	I	-	none observed
Carbonic anhydrase (CA)	red blood cells	I	-	none observed
Alkaline phosphatase (AP)	liver	I	-	none observed
Alcohol dehydrogenase (ADH)	liver	I	never resolved	?

Resolution Symbols: + = consistent results
0 = staining technique did not always give results
- = staining technique rarely gave results.

These patterns are similar to patterns described for rainbow trout by Williscroft and Tsuyuki (1970), Bailey et al. (1970) and Northcote et al. (1970).

The comparison of parent variant types for EST, LDH, TO and MDH enzymes with the progeny of selected crosses (Table 2) indicates that these characteristics were inherited in a predictable manner: each observed protein variant represents a specific genotype determined by two different alleles. Similar results were obtained by Allendorf (1973) for the LDH, TO and MDH systems in rainbow trout.

Table 2. Observed LDH, EST, TO and MDH genotype distributions of progeny from selected matings of Deschutes River rainbow trout.

Parents Genotype	Observed and (Expected) Genotype Distribution			N
	B'B'	B'B	BB	
LDH				
B'B x B'B'	18(20)	22(20)	0(0)	40
B'B x B'B	9(7.5)	19(20)	7(7.5)	35
BB x BB	0(0)	0(0)	20(20)	20
	AA	AB	BB	
EST				
BB x AB	0(0)	19(20)	21(20)	40
AB x AA	19(17.5)	16(17.5)	0(0)	35
	AA	AB	BB	
TO				
BB x AB	19(20)	21(20)	0(0)	40
BB x BB	0(0)	0(0)	26(26)	26
	B'B'	B'B	BB	
MDH				
B'B' x B'B'	40(40)	0(0)	0(0)	40
B'B' x B'B	14(13)	12(13)	0(0)	26

Genotype and gene frequency data (Table 3) show that the greatest differences among the eight groups of trout examined was for the EST and LDH systems. There was a tendency for LDH-BB genotypes to be more frequent in the anadromous type groups. Approximate 95% confidence intervals determined for the most common homozygous genotype of each protein was calculated from: $\frac{p}{n} \pm 1.96 \sqrt{\frac{p(1-p)}{n}}$, where; p is the number of EST-BB, LDH-BB, TO-BB and MDH-B'B' genotypes found in each sample (n). These intervals were:

	EST-BB	LDH-BB	TO-BB	MDH-B'B'
Mainstem rainbow	.25-.51	.09- .31	.84-1.0	.91-1.01
Buck Hollow rainbow	.07-.41	-.01- .25	.75-1.01	.75-1.01
Wild steelhead	.23-.61	.24- .56	.75-1.01	.81-1.03
Bakeoven juveniles	.17-.45	.02- .20	.85-1.01	.90-1.02
Hatchery steelhead	.18-.64	.06- .38	.91- .97	1.0
Residual steelhead	.28-.50	.06- .20	.77- .93	.86- .98
Buck Hollow smolts	.31-.60	.11- .33	.91-1.01	1.0
Jordan juveniles	.37-.55	.94-1.02	.69- .91	.34- .54

In general overlapping confidence intervals among the eight groups of trout were noted for each genotype compared, suggesting that most of the observed differences in genotype frequencies were statistically insignificant. However in a few comparisons the

Table 3. Genotype and gene frequencies for eight groups of Deschutes River steelhead and rainbow trout.

	EST Genotypes				Allele Freq.	LDH Genotypes				Allele Freq.	TO Genotypes				Allele Freq.	MDH Genotypes				Allele Freq.
	AA	AB	BB	N	B	B'B'	B'B	BB	N	B	AA	AB	BB	N	B	B'B'	B'B	BB	N	B
Mainstem Rainbows	.17	.45	.38	50	.61	.28	.52	.20	50	.46	.0	.08	.92	50	.04	.96	.04	.0	50	.02
Buck Hollow Rainbows	.40	.36	.24	25	.42	.32	.56	.12	25	.40	.0	.12	.88	25	.06	.88	.12	.0	25	.06
Wild Steelhead	.27	.31	.42	26	.58	.17	.43	.40	35	.62	.0	.12	.88	25	.06	.92	.08	.0	25	.04
Bakeoven Juveniles	.20	.49	.31	45	.56	.47	.42	.11	45	.31	.0	.07	.93	44	.04	.95	.05	.0	45	.02
Hatchery Steelhead	.12	.47	.41	17	.64	.41	.37	.22	27	.40	.0	.05	.95	18	.02	1.0	.0	.0	21	.00
Residual Steelhead	.24	.37	.39	74	.58	.38	.49	.13	79	.38	.0	.15	.85	73	.08	.93	.07	.0	79	.04
Buck Hollow Smolts	.24	.30	.46	50	.61	.32	.46	.22	50	.45	.0	.04	.96	49	.02	1.0	.0	.0	50	.00
Jordan Juveniles	.24	.35	.41	49	.52	.00	.02	.98	49	.99	.04	.16	.80	49	.12	.90	.10	.0	49	.05

confidence intervals about observed genotype frequencies did not overlap, suggesting that the observed differences in these cases were significant. Specifically, significant differences were found for the comparisons of Wild steelhead vs. Bakeoven juveniles, Wild steelhead vs. Residual steelhead, Jordan juveniles vs. all other groups with respect to LDH-BB genotypes and Jordan juveniles vs. all other groups with respect to MDH-B'B' genotypes.

IV. DISCUSSION

General

The electrophoretic variation of four enzyme systems was examined in eight groups of Deschutes River rainbow trout. The main purpose of this examination was to study the genetic relationships between anadromous and non-anadromous forms of these fish.

Specimen Sampling

Possible problems in obtaining equally representative samples for all eight groups of Deschutes River trout were evident. As noted in Table 3, the number of fish sampled per group ranged from 17 to 79. Sample sizes for hatchery steelhead (17), wild steelhead (26), and Buck Hollow rainbow (25) may be critically small, however this was unavoidable.

It is possible that by sampling each group of fish on a different date an additional source of error was generated. Environmental changes from year to year undoubtedly affect Deschutes River trout populations. Because the "choice" between anadromous and non-anadromous behavior in trout is most likely the result of a variable combination of genetic predispositions and environmental interactions, one could envision this yearly environmental variation to have significant genetic implications.

Genotype-Behavioral Correlations

Omitting the Jordan juvenile group and assuming observed genotype frequency differences are real among the groups tested, there appears to be a positive correlation between the frequency of LDH-BB genotype and the degree to which any group of Deschutes trout was anadromous. Huzyk and Tsuyuki (1973) found a similar correlation with respect to the same enzyme variant for seven rainbow and seven steelhead populations. Most of the rainbow populations they examined had a low frequency of LDH-BB genotypes, whereas the steelhead populations had a much higher occurrence of this particular genotype. Unlike the Deschutes River, the populations which Huzyk and Tsuyuki examined were from coastal streams where both anadromous and non-anadromous forms did not occur together. Therefore, the significance of their findings may not be directly applicable to Deschutes rainbow and steelhead.

Further complicating the adaptation of their generalizations to Deschutes River trout is data that indicates rainbow trout, both anadromous and non-anadromous types, occurring inland of the Deschutes River are genetically different from coastal populations (Allendorf, 1975), particularly with respect to the LDH system. Allendorf (1975) found a higher frequency of LDH-BB genotypes in inland rainbow trout populations than in coastal populations.

If Deschutes River rainbow and steelhead are a somewhat homogeneous group of trout, one way a higher proportion of LDH-BB genotypes may have become characteristic of anadromous forms is through genotype correlated behavior. That is, the behavioral responses of LDH-BB individuals are innately different than the behavioral responses of LDH-BB' or LDH-B'B' individuals.

The mechanism for this phenomenon has been postulated by several authors. Northcote et al. (1970) noted that trout with LDH-B' phenotypes predominated in faster waters, suggesting that these types were physiologically more capable swimmers. The bio-chemical evidence for this difference may be the considerably higher rate at which the LDH-B'_4 protein was observed to convert lactate to pyruvate (Huzyk and Tsuyuki, 1973). It seems likely that aggressive behavior in trout may be related to swimming ability, especially in lotic environments. Chapman (1962) found that coho salmon (Oncorhynchus kisutch) juveniles that were not agressive tended to be displaced downstream within six months when placed in a competitive situation. Under this hypothesis Deschutes River LDH-BB trout would have a lower lactate to pyruvate conversion rate and this lower rate could actually reduce the swimming ability. Therefore their potential for aggressive behavior would be inferior to that of LDH-B'B and LDH-B'B' individuals. LDH-BB individuals, being less aggressive, could be displaced downstream,

eventually to the ocean and become steelhead. Sawyer and Groover[1] have observed that a considerable number of juveniles, probably from steelhead parents, migrate out of Bakeoven Creek four or five months after hatching. These individuals may be displaying downstream migration for the same reason that Chapman (1962) determined nomadic coho salmon (four or five month old coho juveniles) migrated downstream, because of lack of aggressiveness. However this theory is not necessarily a viable point of view. One group, Jordan juveniles, an entirely non-anadromous group, had a high LDH-BB genotype frequency. Any individuals which display downstream migration are immediately swept over several barrier waterfalls. Selection against downstream migration is 100% yet most of the Jordan Creek trout are of genotype which would seem to favor downstream migration under the above hypothesis. Again some of Chapmsn's findings may have relevance here; nomadic coho when placed in a stream aquaria barren of aggressive fish did not show downstream movement. Presently the estimated frequency of LDH-B'B' individuals in Jordan Creek is extremely low, one in 10,000. At these low frequencies the occurrence of a LDH-BB individual being displaced downstream by a more aggressive LDH-B'B' individual would be very rare, perhaps insignificant. This is not to say that Jordan Creek trout are never

[1]Sawyer and Groover, personal communication.

forced downstream (and out of the population) due to lack of aggressive behavior, but merely that the lack of competitive aggressiveness in Jordan Creek trout would not be expected to be related to LDH genotype.

Certainly, high frequencies of LDH-BB genotypes cannot be argued as the single "cause" for a group of trout to display anadromous behavior. However, it is possible that in a trout population highly polymorphic for the LDH-B,B' enzyme system, anadromous behavior is more probable for LDH-BB individuals.

A possible relationship of EST-BB genotype frequencies with anadromous behavior was also found. EST-BB individuals appeared to be more common in anadromous forms. However, this correlation is vague and more groups of trout need to be sampled before any theories can be put forth. Yet it is interesting that the extremes in EST-BB frequencies were observed for a non-anadromous and anadromous group from the same stream, Buck Hollow Creek. Buck Hollow rainbows had an EST-BB frequency of .24, whereas Buck Hollow smolts had an EST-BB frequency of .46 (Table 3). The magnitude of this difference is more than twice that of LDH-BB genotype frequency differences for the same two groups. It appears that EST-BB genotypes may possibly be related to anadromous behavior for trout in Buck Hollow Creek.

Genetic Distinction Among Deschutes River Trout

The primary objective of this study was to genetically compare Deschutes River rainbow and steelhead trout. However, the Deschutes drainage cannot be characterized by a single type of environment or biological system. Therefore, it was necessary to sample non-anadromous and anadromous forms at several locations. It was quite possible that the magnitude of genetic difference between rainbow and steelhead groups was no more significant that the genetic differences among rainbow groups or the genetic differences among steelhead groups.

Throughout this study genotype frequencies have been used as the basis of comparison. It was felt that in many instances gene frequency comparisons would be unreliable because of possible imbalances resulting from environmental selection in diverse environments and genotype correlated behavior. These imbalances usually are reflected by observed genotype frequencies being different from those expected using the Hardy-Weinberg equilibrium hypothesis. For example, two groups of trout sampled from the Deschutes River drainage, Buck Hollow rainbow and hatchery steelhead, were found to have identical LDH-B gene frequencies. Therefore it might be concluded that these two groups are genetically identical with respect to the LDH system. However, not only are the genotypic ratios for both

of these groups out of Hardy-Weinberg equilibrium, but the individual genotype frequencies of Buck Hollow rainbow and hatchery steelhead differ considerably. Assuming that sampling errors were not too great, it is obvious that for the above situation only genotype frequency data is useful in making a distinction between these groups. Similar justifications for using genotype frequencies to make comparisons between populations has also been put forth by Hedrick (1971).

	LDH					
	B'B'		B'B		BB	
	obs.	exp.	obs.	exp.	obs.	exp.
Buck Hollow rainbow	.32	(.36)[2]	.56	(.48)	.12	(.16)
Hatchery steelhead	.41	(.36)	.37	(.48)	.22	(.16)

It was apparent that the observed genetic differences among eight groups of Deschutes River trout was statistically significant in only a few cases. Wild steelhead were significantly different from Bakeoven juveniles and residual steelhead with respect to the LDH-BB frequencies. Although both residual steelhead and Bakeoven juveniles were resident, freshwater groups their origin was known to be partially or totally from anadromous, non-resident parents. Therefore the biological significance of wild steelhead being different from these groups is probably useless for making a general statement that

[2]Numbers in parenthesis indicate expected genotype frequencies according to the Hardy-Weinberg hypothesis.

Deschutes River rainbow and steelhead trout are genetically semi-discrete populations. In addition, the finding of two differences in 84 possible comparisons (Jordan juveniles excluded) is not particularly surprising. In fact, one might anticipate the same results in comparing successive samples from the same population. When one considers the lack of significant genetic difference among the eight groups of trout compared, a rather homogenous single population of non-anadromous and anadromous trout seems like a more plausible hypothesis.

One group, Jordan juveniles, isolated by several barrier waterfalls, was significantly different from all other groups with respect to both MDH-B'B' and LDH-BB genotype frequencies. It is not clear if these unique characteristics resulted from random drift, selection or colonization by populations unlike those found in the Deschutes River.

V. CONCLUSIONS

1. Four proteins were found to be genetically polymorphic in Deschutes River trout populations: LDH, EST, MDH, TO. Two other proteins, IDH and TFN were thought to be highly polymorphic, however consistent resolution of these two systems was not achieved.
2. The greatest genetic variation among the eight groups of trout compared was found for the LDH system.
3. In most comparisons, statistically significant genetic differences were not found among eight groups of Deschutes River trout.
4. The lack of consistent statistically significant differences between Deschutes River anadromous and non-anadromous groups suggests that an overall genetic distinction between these two forms, based on the comparison of four enzyme loci (LDH, EST, MDH and TO), is unlikely.
5. Jordan Creek trout appear genetically distinct from all other groups of Deschutes trout studied.

VI. MANAGEMENT IMPLICATIONS

1. The genetic results obtained in this study are supportive evidence that considerable interbreeding and mixing of gentic material occurs between Deschutes River rainbow and steelhead trout.
2. Jordan Creek contains a genetically unique population. All efforts should be made to preserve this population in its native state. Particularly detrimental would be introduction of non-indigenous stocks of fish. Other Deschutes tributary streams may also have genetically unique trout populations above barrier water falls. Similar management practices are recommended for these as well.

VII. FURTHER STUDY

1. Refinement of electrophoretic techniques and a larger sampling effort would result in a more precise genetic discrimination among groups of Deschutes River trout.
2. An intensive genetic study of Deschutes tributary trout populations existing above barrier waterfalls may provide some interesting genetic and evolutionary findings.

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APPENDIX

Table 1. Description of electrophoresis buffer systems used in examining Deschutes River rainbow trout for protein variants.

Buffer System I (Ridgeway et al. 1970)

Gel Buffer (pH 8.5)
Tris (.03 M)
citric acid (.005 M)
1% electrode buffer by total volume

Electrode Buffer (pH 8.1)

lithium hydroxide (0.06 M)
boric acid (0.3 M)

Buffer System II (Wolf et al. 1970)

Gel Buffer (pH 6.5)

1:10 dilution of electrode buffer

Electrode Buffer (pH 6.5)

4 parts dibasic sodium phosphate (0.1 M)
6 parts monobasic sodium phosphate (0.1 M)

Table 2. Description of staining mixtures used for detection of 16 different proteins in Deschutes River rainbow trout.

Enzyme	Ingredients	Procedure Comments	Reference
Lactate dehydrogenase (LDH)			
	70 ml H_2O 15 ml 1.0 M Tris (pH 7.1) 5 ml 1.0 M KCN 10 ml 1.0 M lactic acid 50 mg NAD^+ 30 mg NBT 3 mg PMS	Adjust to pH 7.0, then add PMS, incubate in dark.	E-C App. Corp. (1971)
Malate dehydrogenase (MDH)			
	Identical to LDH except add 80 ml H_2O and 1.34 g malic acid	Same as LDH	E-C App. Corp. (1971)
Tetrazolium oxidase (TO)			
	90 ml H_2O 10 ml 1.0 M Tris-HCl (pH 7.1) 40 mg MTT 10 mg PMS	Adjust pH to 8.0, incubate in light.	Modified from Shaw and Prasad (1970)
Esterase (EST)			
	100 mg Fast Blue RR 50 mg α-Naphthyl acetate 2.5 ml acetone 2.5 ml H_2O 10 ml Tris-HCl (7.1) 85 ml H_2O	Dissolve Fast Blue RR and α-Naphthyl acetate in accetone before adding the other ingredients.	Shaw and Prasad (1970)
Isocitrate dehydrogenase (IDH)			
	20 mg $NADP^+$ 20 mg NBT 3 mg PMS 138 mg $Na_3 \cdot$ isocitrate $\cdot$ 4 H_2O 90 ml H_2O	Same as LDH	E-C App. Corp. (1971)
Alphaglycerophosphate dehydrogenase (AGPDH)			
	Same as LDH but substitute 1 M alphaglycerophosphate for lactic acid.	Same as LDH	E-C App. Corp. (1971)

Table 2. Continued.

Enzyme	Ingredients	Procedure Comments	Reference
Transferrin (Tfn), Note: nonspecific general protein stain			
	0.1% Nigrosin Buffalo Black Solution	Stain is removed after 20 min. and the gel is subjected to several washings with a 1:4:5 acetic acid, methanol water solution.	Utter et al. (1970)
Galactose dehydrogenase (GalDH)			
	Same as LDH but use 10 ml of 1 M Galactose instead of lactic acid.	Same as LDH	E-C App. Corp. (1971)
Sorbitol dehydrogenase (SDH)			
	85 ml H_2O 5 ml 40% Sorbitol 10 ml Tris-HCl (pH 7.1)	Same as LDH	Shaw and Prasad (1970)
Aspartate aminotransferase (AAT)			
	100 ml H_2O 75 mg α-ketoglutaric acid 225 mg aspartic acid 3 g NeH_2PO_4 1 g PUP 1 g Fast Garnet GBC salt		Johnson et al. (1972)
Phosphoglucomutase (PGM)			
	90 ml H_2O 10 ml 1.0 M Tris-HCl (pH 7.1) 600 mg $Na_2 \cdot$ glucose-1-phosphate $\cdot 4H_2O$ 10 mg $NADP^+$ 80 units glucose-6-phosphate dehydrogenase 1 mg PMS 20 mg NBT	Same as LDH	E-C App. Corp. (1971)

Table 2. Continued.

Enzyme	Ingredients	Procedure Comments	Reference
Succinate dehydrogenase (SucDH)			
	100 ml H_2O 1.456 g sodium succinate 65 mg DPN 50 mg ATP 5 mg PMS 35 mg NMT .370 g EDTA .320 g K_2HPO_4	Same as LDH	Brewer (1970)
Alkaline phosphatase (AP)			
	100 ml H_2O 50 mg sodium α-napthyl phosphate 500 mg Blue RR salt 1.74 g NaCl 0.121 g Tris	Adjust pH to 8.5	Brewer (1970)
Carbonic anhydrase (CA)			
	0.1% Bromthymol Blue	Cover gel surface for 15 min. with filter paper soaked in Bromthymol Blue. Remove paper and direct CO_2 through rubber tube onto surface of gel.	Brewer (1970)
Alcohol dehydrogenase (ADH)			
	Same as LDH, except use 77 ml H_2O and substitute lactic acid with 3 ml 95% ethanol	Same as LDH	E-C App. Corp. (1971)