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GENE DUPLICATION WITHIN THE FAMILY SALMONIDAE: II. DETECTION AND DETERMINATION OF THE GENETIC CONTROL OF DUPLICATE LOCI THROUGH INHERITANCE STUDIES AND THE EXAMINATION OF POPULATIONS

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ABSTRACT. We examine the interpretation of electrophoretic data reflecting duplicated gene loci in salmonid fishes. Genetic models are considered to explain electrophoretic patterns of proteins reflecting duplicated genes where common alleles give rise to proteins of identical electrophoretic mobilities. It is shown that in the absence of breeding data it is impossible to distinguish between a model of tetrasomic inheritance and one of disomic inheritance where alleles segregating at two loci occur at the same frequency. However, it is shown that disomic inheritance can be verified by the examination of population phenotypic distributions under certain conditions. Family data are presented verifying disomic inheritance of malate dehydrogenase in rainbow trout (*Salmo gairdneri*) and aspartate aminotransferase in chum salmon (*Oncorhynchus keta*). Examination of 19 salmonid biochemical systems indicate that 8 systems clearly reflect gene duplication, 8 do not, and 3 systems lack variation, precluding detection of gene duplication; genetic variants at all of the duplicated loci appear to segregate disomically. No significant evidence for genetic linkage was found among 10 jointly segregating pairs of loci in rainbow trout and one pair of loci in chum salmon; one comparison in rainbow trout (IDH-AGPDH) approached significance (P=.06).

INTRODUCTION

Measuring the patterns and amounts of genetic variation in natural populations of many species is presently a major thrust of experimental population genetics. This examination has only been possible since the development of the electrophoretic separation of proteins as a means of examining the

gene products of many individual loci. Since 1968, our group has conducted an extensive survey of genetic variation in populations of fish and marine invertebrates. Our efforts have been concentrated somewhat on the salmonid fishes because of our interest in the evolutionary implications of their extensive gene duplication and their usefulness in inheritance studies. (The ripe sex products from both sexes can be artificially removed and stored under refrigeration while the parental types are determined electrophoretically prior to selecting the desired matings.)

The efforts of one author (Allendorf) have been concentrated on an intensive survey of genetic variation in populations of *Salmo* species with particular emphasis on the rainbow trout (*S. gairdneri*). The extensive gene duplication found in salmonids often confounds the genetic interpretation of the observed variation. Because of the critical importance of understanding this genetic basis we are involved in a series of inheritance studies directed toward the identification of the genetic control of polymorphic loci in salmonids.

The initial electrophoretic studies with salmonids revealed additional loci coding for lactate dehydrogenase (LDH) in comparison with other vertebrate species (Morrison and Wright, 1966). Subsequent studies of salmonids have revealed similar gene duplications for a number of other enzymes--e.g. malate dehydrogenase (Bailey et al., 1970) and isocitrate dehydrogenase (Wolf et al., 1970). Ohno has postulated that the salmonids are descended from a recent tetraploid ancestor based upon this gene duplication, relative DNA contents, and chromosomal characteristics (Ohno et al., 1969). Although Ohno's theory is sound and based upon valid evidence, some subsequent attempts to reinforce this theory have not been sound (see Allendorf and Utter, 1973). Upon reading the relevant literature one is left with the impression that virtually all enzymes in salmonids are coded for by duplicate loci and that many of these loci are segregating tetrasomically. Our studies are in conflict with this impression. We have found no evidence for the duplication of many protein loci and we have found no indication of tetrasomic inheritance even for those loci which have been reported to be inherited tetrasomically (this in no way rules out the existence of tetrasomic loci in salmonids but rather points out that they have not yet been detected).

The purpose of this present paper is to examine the interpretation of duplicate loci detected electrophoretically and to review our genetic investigations of rainbow trout in view of the accepted level of gene duplication and

patterns of inheritance of salmonid loci. The evolutionary question to be considered in the course of these studies is the fate of duplicated loci. We feel that the salmonids represent an outstanding opportunity to examine this question experimentally.

DETERMINATION OF GENETIC CONTROL OF DUPLICATED LOCI

The multiple loci coding for many salmonid proteins often make the interpretation of genetic control rather difficult. The disagreements which can result are illustrated by the reported number of loci coding for LDH in salmonids; values all the way from five to eight have been reported in the literature (Massaro and Markert, 1968).

We will examine a hypothetical case of a single enzyme and outline some of the difficulties in determining the exact nature of genetic control of that enzyme. We will restrict our example to the case of a maximum of two disomic loci (or a single tetrasomic locus) with two alleles (A and A'). Table 1 outlines possible genetic models controlling these loci, while Figure 1 presents typical electrophoretic phenotypes for a dimeric enzyme in this hypothetical case. Two basic questions are to be examined: (1) Is there any evidence of gene duplication? and (2) If there has been gene duplication, is there a single tetrasomic locus or two disomic loci?

PHENOTYPIC DISTRIBUTIONS IN POPULATIONS

Predictions of the expected phenotypic distributions for each of the models can be made assuming Hardy-Weinberg proportions--see Table 2. Certain conclusions can be drawn pertaining to the nature of genetic control involved by comparing the observed distributions within populations with these theoretical distributions.

Models A, C, and G all predict a single-banded phenotype to be found in all individuals of a population because of the lack of polymorphism. Therefore, no conclusions of genetic control can be made if this distribution is found in a population. However, model D which also lacks polymorphism predicts a fixed multi-banded phenotype to be found in all individuals. If this "fixed heterozygote" situation is found in a population it is best explained by two monomorphic, disomic loci fixed for alleles with differing electrophoretic mobilities.

Model B is also distinguishable in that there are no asymmetrical banded phenotypes and the single banded alter-

TABLE 1
List of the possible genetic models to be considered that could be controlling a protein system examined electrophoretically. (P = frequency of allele A; Q = frequency of allele A'.)

Genetic Model	Disomic Loci		Tetrasomic Loci		Allele Frequencies
	Mono-morphic	Poly-morphic	Mono-morphic	Poly-morphic	
A	1	0	0	0	$p=1$
B	0	1	0	0	p
C	2	0	0	0	$p_1=p_2=1$
D	2	0	0	0	$p_1=1, p_2=0$
E	1	1	0	0	$p_1=1, p_2$
F	0	2	0	0	p_1, p_2
G	0	0	1	0	$P=1$
H	0	0	0	1	P

nate homozygote type is found at a much higher frequency than under any of the other models.

Models E, F, and H are not so easily distinguished. In fact, Models F (two polymorphic disomic loci) and H (a single polymorphic tetrasomic locus) predict identical phenotypic distributions when $q_1=q_2$ for model F. Therefore, the existence of tetrasomic inheritance can never be conclusively demonstrated by examining the phenotypic distribution in a population since a disomic model can always fit the data equally well. When there is a comparatively high amount of variation model E is distinguishable from models F and H by virtue of the absence of phenotypes four and five. Accordingly, when the amount of variation is low these models are not easily distinguished. It is interesting to examine the predicted phenotypic distributions of these three models as shown in Figure 2. The expected Hardy-Weinberg proportions of a population are represented by the family of values between these two curves in the case where the correct model cannot be distinguished. Similarly, if Model F is shown to be the correct one (through inheritance studies as outlined

TABLE 2

Expected Hardy-Weinberg proportions for each of the eight genetic models considered as outlined in Table 1.

Duplicated Phenotype	Non-Dupl Phenotype	Expected Phenotypic Distributions / Genetic Model A	B	C	D	E	F	G	H
A_4	A_2	1	p^2	1	0	p_2^2	$p_1^2p_2^2$	1	P
A_3A'	---	0	0	0	0	$2p_2q_2$	$2p_1^2p_2q_2+2p_1p_2^2q_1$	0	$4P^3Q$
$A_2A'_2$	AA'	0	2pq	0	1	q_2^2	$p_1^2q_2^2+4p_1p_2q_1q_2+p_2^2q_1^2$	0	$6P^2Q^2$
AA'_3	---	0	0	0	0	0	$2p_1q_1q_2^2+2p_2q_1^2q_2$	0	$4PQ^3$
A'_4	A'_2	0	q^2	0	0	0	$q_1^2q_2^2$	0	Q^4

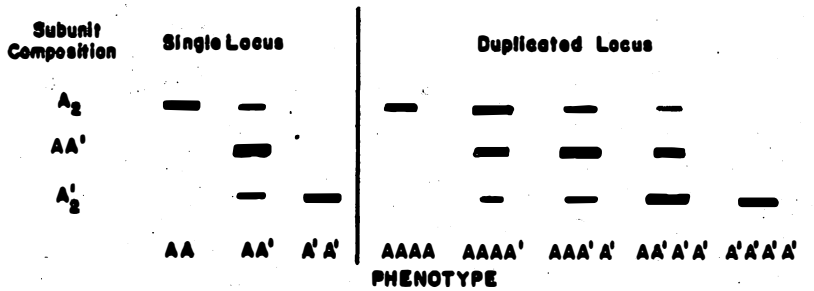

Figure 1. Diagrammatic representation of typical electrophoretic phenotypes for a dimeric enzyme controlled by either a single locus or a duplicated locus.

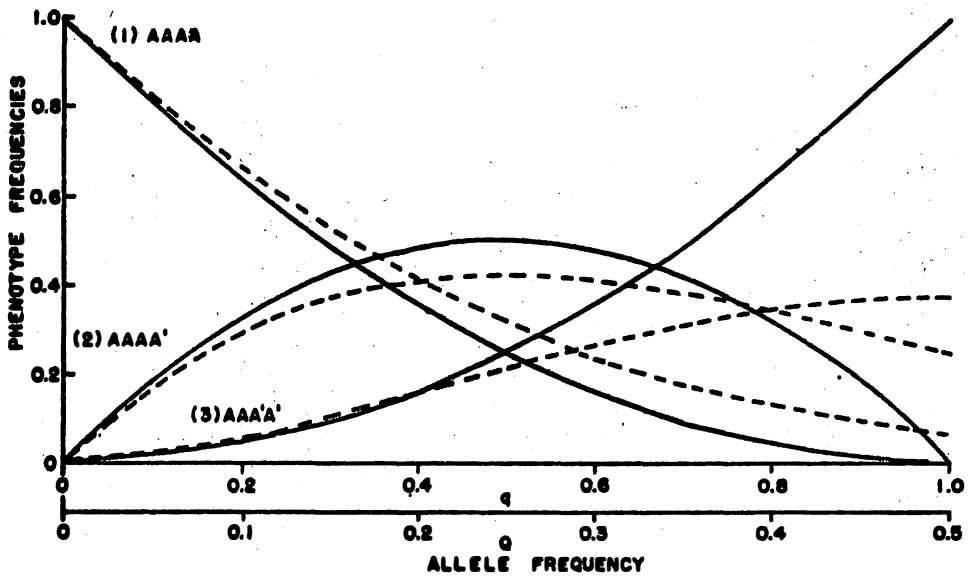

Fig. 2. Graphical representation of expected phenotypic distributions for Model E(solid line) and Models F and H (dotted line) when $q_1=q_2$ under Model F. Upon examining a population, the overall allele frequency (Q) of the A' allele can be determined by dividing the total number of A' alleles observed by 4N (where N=number of individuals examined). This frequency is the allele frequency for a tetrasomic locus

Legend for Fig. 2, continued:
(Q) and is also equal to both q_1 and q_2 ($Q=q_1=q_2$) under Model F when $q_1=q_2$. This frequency is related to q_2 from Model E by the equation $q_2=2Q$. This graph demonstrates the differences in expected Hardy-Weinberg proportions for a duplicated locus for these three cases; (1) a single polymorphic locus, (2) both loci polymorphic and (3) a single tetrasomic locus. When $q_1 \neq q_2$ for case (2) the expected Hardy-Weinberg proportions lie between these curves.

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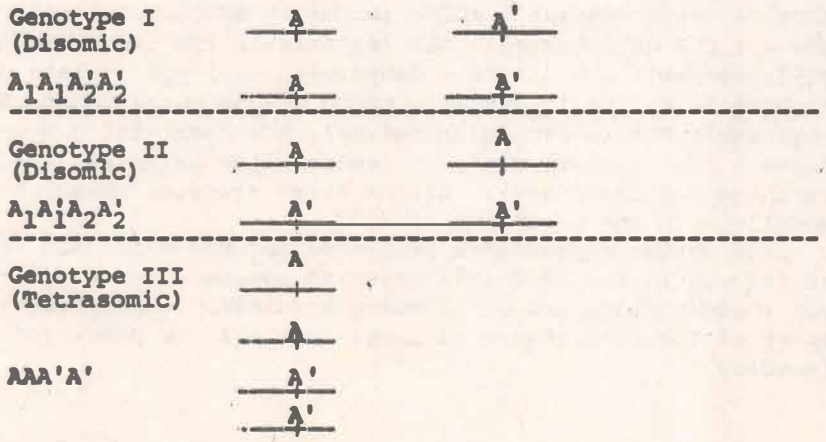
in the next section), the expected Hardy-Weinberg proportions are represented by this same family since gene frequencies cannot be assigned to individual loci.

The major conclusions that can then be drawn from this section are that (1) tetrasomic inheritance cannot be conclusively demonstrated only by the phenotypic distribution of a population and (2) under certain conditions, disomic inheritance can be demonstrated in this way.

INHERITANCE EXPERIMENTS

The mode of inheritance of duplicated loci (either disomic or tetrasomic can be positively verified through inheritance studies. Individuals with single doses of a variant allele do not provide useful information since their gametes are expected to segregate 1:1 (AA : AA') under both a tetrasomic and disomic model. However, individuals with a double dose of a variant allele do provide sufficient information to distinguish between these models.

Examination of the gametic output from an individual typed as AAA'A' will provide the desired information. The phenotype AAA'A' can represent any one of three genotypes.



These genotypes and their expected gametic ratios are presented in Table 3. It can be seen that each genotype predicts a unique gametic distribution. In the disomic case, the proportion of genotypes I and II reflect the gene frequencies p_1 and p_2. The gametic ratios for genotypes II and III are variable because of possible linkage in the disomic model and possible double reduction division in the tetrasomic model. Therefore, to verify disomic inheritance, parental types corresponding to both genotypes I and II should be observed. In addition, it should be noted that examining the gametic ratios from genotype II will also provide a measure of possible linkage between the two loci.

MATERIALS AND METHODS

The results presented here are based on the examination of thousands of rainbow trout from numerous populations as well as from a continuing series of experimental matings. We have examined both non-anadromous and anadromous (steelhead) populations of *S. gairdneri*, and have found no reason for treating these two forms as being distinct. For purposes of this paper, we will use the common name rainbow trout to represent both the anadromous and non-anadromous forms.

Our methods have been documented elsewhere (Utter et al., 1973a). Three buffer systems were used: (1) a discontinuous system described by Ridgway et al., (1970), (2) a continuous tris-borate-EDTA system (pH 8.6) described by Markert and Faulhaber (1965), and (3) a continuous phosphate system (pH 6.5) described by Wolf et al., (1970).

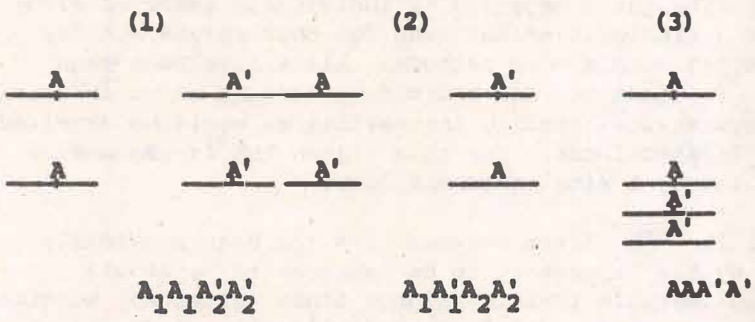
The following proteins were examined in the course of these studies: AAT (aspartate aminotransferase); ADH (alcohol dehydrogenase); AGPDH (alphaglycerophosphate dehydrogenase); DIA (diaphorase); EST (esterase); IDH (isocitrate dehydrogenase); LDH (lactate dehydrogenase); MDH (malate dehydrogenase); ME (malic enzyme); 6PGDH (6-phosphogluconate dehydrogenase); PGM (phosphoglucomutase); SDH (sorbitol dehydrogenase); TFN (transferrin); TO (tetrazolium oxidase); and XDH (xanthine dehydrogenase). All of these proteins migrated anodally with the exception of ADH.

The mating experiments presented for MDH-B and AAT were carried out in the 1973-1974 spawning season. The progeny from these matings are still being examined. A more detailed report of the inheritance of these loci will be presented elsewhere.

TABLE 3

Comparison of disomic verus tetrasomic inheritance in an individual carrying two doses of a variant allele.

PARENTAL GENOTYPE



COMPARATIVE SEGREGATION RATIOS OF GAMETES

Gamete Genotype	Parental Genotype (I)	(II)*	(III)**
AA	0	1	1
AA'	1	2	4
A'A'	0	1	1

* Assuming no linkage.

** Assuming chromosome segregation.

RAINBOW TROUT PROTEIN LOCI

DIA, 6PGDH, XDH. These enzymes were found to be represented by a single invariant band in all individuals examined. Since this result is in agreement with models A, C, and G, no conclusions can be made as to whether they are coded for a single locus or duplicated loci.

ME. Two forms of this enzyme were observed--one which was predominant in muscle extracts and one which was predominant in liver extracts. Both of these forms were uniformly seen as a single band. As explained above, the number of loci coding for each form could therefore not be determined.

ADH. The great majority of individuals examined also displayed a single invariant band for this enzyme. A few variant types with a more cathodal allele have been seen however. In these variant individuals, there is no indication of any asymmetrical banding intensities as would be expected for a duplicated locus. For this reason ADH is assumed to be coded for by a single disomic locus.

PGM, TO, TFN. These enzymes have all been previously reported by our laboratory to be represented by single disomic polymorphic loci in rainbow trout (Utter and Hodgins, 1972). A report of possible gene duplication of TO (Cederbaum and Yoshida, 1972) was found to be in error (Utter et al., 1973b).

LDH. Our previously reported results (Utter et al., 1973a) indicate five loci coding for LDH in salmonids. This enzyme is an example of fixed heterozygosity in that all five loci have common alleles with differing mobilities. The duplicated LDH-B loci present an especially interesting evolutionary history. In addition to evolving common alleles of different mobilities, these loci have also evolved differential tissue specificity. Only the B_2 locus is expressed in liver tissue while the B_1 locus is strongly predominant in heart tissue.

Although LDH is the classic example of duplicate loci in salmonids, not all salmonid LDH loci display gene duplication. The eye form of LDH is present in many families of fish and is presumed to be the result of a single evolutionary event (Horowitz and Whitt, 1972). This indicates that the LDH eye form was present in the salmonid lineage before the presumed polyploid event. However, genetic evidence has been presented which shows the salmonid eye LDH to be coded by a single locus (Morrison and Wright, 1966; Wright and Atherton, 1970).

AGPDH. Previous publications from our group have reported this enzyme to be represented by a single polymorphic disomic locus (Utter and Hodgins, 1972). Examination of this enzyme with buffer system (3) clearly revealed the

presence of an additional locus not detectable with buffers (1) or (2). The gene products of this second locus do interact with the products of the first locus in that the appropriate heterodimers are formed. This additional locus is fixed for an allele with a different mobility than both alleles seen at the first locus, thereby creating a fixed heterozygote effect.

EST. A single polymorphic disomic locus predominantly expressed in liver extracts has been observed. Inheritance studies have confirmed the genetic basis of this variation but there are indications of possible ontogenetic and environmental effects on the expression of this locus. There is no indication of duplication of this locus.

SDH. This enzyme has been reported to be tetrasomically inherited in rainbow trout on the basis of the observed phenotypic distribution in a single population (Engel et al., 1970). As shown in a prior section, tetrasomic inheritance cannot be verified in this manner. We have found multiple banded phenotypes in all rainbow trout examined in accordance with model D, indicating this enzyme to be coded for by two disomic loci with common alleles of different mobilities. We have seen genetic variation at low frequency for this enzyme but have not found it in the populations used for the mating experiments. However, we do have preliminary inheritance data for a similar variant found in cutthroat trout (*S. clarki*) which verifies this interpretation.

IDH. The supernatant form of this enzyme (IDH-s) has also been reported to be inherited tetrasomically in rainbow trout on the basis of the phenotypic distribution in a population (Wolf et al., 1970). In an inheritance study previously reported (Allendorf and Utter, 1973), we have shown this variation to be controlled by two disomic loci.

The mitochondrial form of this enzyme (IDH-m), as best seen in muscle extracts, is represented by three nonvariant bands indicating the presence of two monomorphic disomic loci with common alleles of different mobilities.

MDH-B. Bailey et al., (1970) presented an excellent biochemical and genetic analysis of salmonid MDH demonstrating the existence of duplicate loci coding for the B form. However, since all of their matings involved individuals with at most a single dose of the variant allele they could not distinguish between disomic and tetrasomic inheritance. We have recently made the necessary crosses to answer this

TABLE 4

Inheritance of duplicate loci. In each case only the results from one family are presented here. Additional families (some with parental types corresponding to genotype 1 in Table 3) further confirm these conclusions.

(a) Rainbow Trout MDH-B

PARENTAL PHENOTYPES		PROGENY PHENOTYPES OBSERVED (DISOMIC) /TETRASOMIC/ BBBB	BBBB'	BBB'B'	X^2	d.f.		P
BBBB	BBB'B'	80	165	70				
		(78.8)	(157.5)	(78.8)	1.36	2	>	0.5
		/52.5/	/210.0/	/52.5/	33.36	2	<	0.0001

(b) Chum Salmon AAT

PARENTAL PHENOTYPES		PROGENY PHENOTYPES OBSERVED (DISOMIC) /TETRASOMIC/ AAAA	AAAA'	AAA'A'	X^2	d.f.		P
AAAA	AAA'A'	44	85	43				
		(43.0)	(86.0)	(43.0)	0.03	2	>	0.9
		/28.7/	/114.7/	/28.7/	27.80	2	<	0.0001

TABLE 4, continued

Conclusions for both cases:

(1) Inherited disomically

(2) Parental genotypes - (a) $B_1B_1B_2B_2 \times B_1B'_1B_2B'_2$

(b) $A_1A_1A_2A_2 \times A_1A'_1A_2A'_2$

(3) No indication of linkage

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question. Table 4 outlines the results from one of these crosses. There are clearly two disomic loci both of which are polymorphic in the population we examined. Additional results from other families confirmed this conclusion. In addition, Table 4 examines possible linkage between the two loci by comparing the frequencies of the two linkage classes (BBBB + BBB'B' : BBBB'). There is no indication of linkage on this basis.

MDH-A. Bailey et al., (1970) presented some evidence for the duplication of this locus in brown trout (*S. trutta*) and subsequent evidence has supported this conclusion (Bailey, personal communication). We have seen a variant of this form at a low frequency in some rainbow trout populations. Based on the relative intensities of bands in the variant phenotypes it appears that this locus is not duplicated in rainbow trout. Additional evidence in the form of inheritance data is desirable before it can be firmly concluded that this enzyme is coded for by different number of loci in these two closely related species.

AAT. The common phenotype found in rainbow trout for this enzyme is a single distinct band. There is a low frequency variant found in some populations. The intensities of bands found in the variant phenotypes are typical of a duplicated locus. Inheritance studies have not been carried out for this enzyme with rainbow trout. However, inheritance studies have been done for this enzyme with the chum salmon (*Oncorhynchus keta*). The results from one family from these experiments are shown in Table 4. Comparison with the expected disomic and tetrasomic ratios indicate that chum salmon AAT is controlled by two polymorphic disomic loci.

As with rainbow trout MDH-B loci, there is no indication of linkage between these duplicated loci. One cannot conclude on this basis that a similar mode of genetic control exists in rainbow trout. However, these data directly apply to the question of genetic control of duplicated loci in salmonids.

JOINT SEGREGATION OF LOCI

Whenever possible in the course of our inheritance experiments, we have tested for linkage between segregating loci by testing for any aberrant 2-way joint segregation in progeny from double heterozygote individuals. The results of these tests are summarized in Table 5. There is no definite indication of linkage. In the case of IDH - AGPDH, the deviation from random segregation did approach significant proportions ($P > .06$). Unfortunately, it has not been possible to examine further the joint segregation of these loci.

Of particular interest is the joint segregation of duplicated loci. Aberrant joint segregation of duplicated LDH loci in both brook trout (*Salvelinus fontinalis*) and rainbow trout have been reported (Morrison, 1970; Davisson et al., 1973). Recently, Aspinwall (1974) has reported limited data indicating aberrant joint segregation of duplicated MDH loci in pink salmon (*O. gorbuscha*). Our results with MDH-B in rainbow trout and AAT in chum salmon do not indicate any abnormal joint segregation of these duplicated loci in these species.

SUMMARY

Table 6 presents a summary of the rainbow trout loci we have examined. Contrary to the statements of some authors, a significant portion of loci in this salmonid species show no evidence of gene duplication. If we accept the tetraploid origin of salmonids, these loci then seem to represent evidence of Haldane's (1933) original suggestion that if a gene is duplicated one of the two resulting genes may become nonfunctional because of the fixation of a deleterious mutation. One other possible fate of a duplicated gene is the evolution of differential function as demonstrated by the duplicated LDH-B loci which have evolved differential tissue specificity.

We have found no evidence of tetrasomic inheritance in salmonids. We have also shown that previous reports of tetrasomic inheritance in salmonids are not sound. These results do not, of course, rule out the existence of tetrasomic loci in salmonids, but rather are meant to show that there is

TABLE 5
Joint segregation ratios of rainbow trout (and chum salmon) loci.

Loci		Linkage Class I	Linkage Class II	Chi-square*
$MDH\text{-}B_1$	IDH-3	62	55	.42
		34	23	2.12
TO	IDH-3	69	50	3.03
		52	46	.37
$MDH\text{-}B_1$	TO	40	53	1.82
		71	76	.17
		89	79	.60
$MDH\text{-}B_1$	AGPDH-1	73	72	.01
$LDH\text{-}B_2$	IDH-3	66	59	.39
$LDH\text{-}B_2$	TO	53	57	.15
		86	74	.90
		100	108	.31
$LDH\text{-}B_2$	$MDH\text{-}B_1$	53	44	.84
		48	52	.16
		121	127	.15
TO	AGPDH-1	70	79	.54
IDH-3	AGPDH-1	49	70	3.71
$MDH\text{-}B_1$	$MDH\text{-}B_2$	150	165	.71
**AAT-1	AAT-2	85	87	.02

* - d.f. = 1
** - Chum salmon

TABLE 6
Summary of rainbow trout loci examined.

Protein	Duplicated?	Loci	Mode of inheritance (if duplicated)
AAT	Yes	AAT-1	(Disomic in chum salmon)
		AAT-2	
ADH	No	ADH	
AGPDH	Yes	AGPDH-1	Disomic
		AGPDH-2	
DIA	?	DIA	
EST	No	EST	
IDH(m)	Yes	IDH-1	Disomic
		IDH-2	
IDH(s)	Yes	IDH-3	Disomic
		IDH-4	
LDH-A	Yes	LDH-A_1	Disomic
		LDH-A_2	
LDH-B	Yes	LDH-B_1	Disomic
		LDH-B_2	
LDH-C	No	LDH-C	
MDH-A	No	MDH-A	
MDH-B	Yes	MDH-B_1	Disomic
		MDH-B_2	
ME	No	ME-1	
		ME-2	
6PGDH	?	6PGDH	
PGM	No	PGM	

TABLE 6, continued:

	Duplicated?	Loci	Mode of inheritance (if duplicated)
SDH	Yes	SDH-1 SDH-2	Disomic
	No	TFN	
	No	TO	
	?	XDH	

at present no evidence for tetrasomic inheritance in salmonids.

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