# GENETIC ANALYSIS OF DUPLICATE MALATE DEHYDROGENASE LOCI IN THE PINK SALMON, *ONCORHYNCHUS GORBUSCHA*

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ABSTRACT

The results of breeding experiments with the pink salmon, *Oncorhynchus gorbuscha*, indicate that s-MDH-A and s-MDH-B subunits are each encoded by duplicate loci. Limited evidence suggests also that the two loci encoding for the s-MDH-A subunit are each polymorphic and linked or pseudolinked.

SUPERNATANT malate dehydrogenase (s-MDH) has been studied in a diverse group of vertebrates from agnathans to man. Such studies have revealed that MDH exists as a dimeric molecule (DAVIDSON and CORTNER 1967; BAILEY *et al.* 1970) with a molecular weight of about 60,000 (MURPHEY *et al.* 1967). The number of genes encoding for s-MDH in vertebrates may vary. In reptiles, birds, and mammals s-MDH typically exists as a single major anodal form (sometimes other lesser bands are also present) (KITTO and WILSON 1966; DAVIDSON and CORTNER 1967; KARIG and WILSON 1971) which suggests single gene control. In fishes and amphibians three equally spaced anodal bands of s-MDH are commonly observed (BAILEY *et al.* 1970). Presumably, these bands in most cases are produced by two loci encoding for different s-MDH subunits (designated A and B). WHEAT, WHITT and CHILDERS (1972) have demonstrated that s-MDH-A and s-MDH-B in bluegill sunfish-red-ear sunfish hybrids (*Lepomis macrochirus* × *Lepomis microlophus*) are controlled by two unlinked loci. The salmonid fishes are an interesting exception in that more than two s-MDH loci are present in those species studied. BAILEY *et al.* (1970) have demonstrated the presence of duplicate loci encoding for the s-MDH-B subunit of the king salmon, *Oncorhynchus tshawytscha*, and have provided considerable evidence for duplicate genes encoding for the s-MDH-A subunit in the brown trout, *Salmo trutta*. This and other evidence, e.g. DNA measurements and chromosome studies, led OHNO (1970) and other workers to the conclusion that the salmonids are tetraploids.

In this communication breeding experiments were conducted with another salmonid, the pink salmon, *Oncorhynchus gorbuscha*, in order to demonstrate the presence or absence of duplicate loci for both s-MDH-A and s-MDH-B in a single salmonid species. In addition, an attempt was made to determine the linkage relationships between any multiple loci.

## MATERIALS AND METHODS

### *Breeding Experiments*

In August of 1971, ova, sperm, and muscle samples of pink salmon were collected from adults gill-netted in Fish Creek near Juneau, Alaska. The ova or sperm of individual fish were packaged separately, without water, in plastic bags and were stored in an insulated cooler surrounded with crushed ice. The muscle samples were wrapped with plastic sheeting (Saran Wrap) and aluminum foil, and frozen with dry ice. The gametes and muscle samples were subsequently shipped to Saint Louis University where the breeding experiments were performed.

Prior to the breeding experiments the MDH phenotype of each adult was determined by horizontal starch-gel electrophoresis as described below. The ova and sperm of parents with particular MDH phenotypes were combined, and the resulting zygotes were incubated in flowing spring water (ca. 14°) until they reached the fry stage (88 days). Approximately 30 hours elapsed between the time of gamete collection and fertilization. The resulting pink salmon offspring were also frozen prior to electrophoretic analysis.

### *Electrophoresis*

Muscle samples of parents and progeny were homogenized in low ionic strength (0.05 M) phosphate buffer (pH 8.5). The homogenate was centrifuged at 39,000 g for 15 minutes. Horizontal starch-gel electrophoresis of the supernatant was conducted with the apparatus of TSUYUKI *et al.* (1966). Difficulties were encountered in selecting a single buffering system appropriate for the identification of MDH phenotypes possessing both A′ and B′ subunits simultaneously. Consequently, two systems were employed in the analysis of each fish. For identifying MDH phenotypes which possess A′ subunits a procedure slightly modified after BAILEY and WILSON (1968) was employed. Electrophoretic separation was conducted for 6 hours at 100 V which employed a citrate-phosphate gel buffering system containing 12% hydrolyzed potato starch (Connaught Laboratories). For MDH phenotypes possessing B′ subunits best results were obtained with electrophoresis conducted for 2¾ hours at 180 volts employing a Tris-citrate buffer (pH 6.8). The bridge buffer contained 0.15 M Tris and 0.05 M citric acid; the gel buffer was 0.075 M Tris and 0.025 M citric acid. The gel in this system also contained 12% hydrolyzed starch.

After electrophoresis, the gels were stained for MDH by immersion in 200 ml 0.1 M Tris (pH 8.5) which contained 30 mg nicotinamide adenine dinucleotide, 1 gm L-malic acid, 30 mg nitro blue tetrazolium, and 5 mg phenazine methosulfate.

## RESULTS

### *Typical s-MDH Phenotypes*

Examination of pink salmon adults from Fish Creek revealed numerous s-MDH phenotypes (Figure 1). The most prevalent phenotype was one in which three equally spaced anodal isozymes were seen (Figure 1a,c,e). The common pink salmon phenotype appears to correspond to the one of the king salmon as described by BAILEY *et al.* (1970). Although I have not compared directly the common s-MDH phenotype between the pink salmon and the king salmon, the examination of s-MDH in the sockeye salmon, *O. nerka*, served as a common denominator between the studies of BAILEY *et al.* (1970) and the present study. BAILEY *et al.* (1970) found that king salmon and sockeye salmon have a similar common s-MDH phenotype. In the present study, the similarity between the common s-MDH phenotype of the pink salmon and sockeye salmon was discovered. It was concluded, therefore, that the common s-MDH phenotype of the pink salmon and king salmon are similar. Consequently, the three s-MDH isozymes of the common pink salmon phenotype (Figure 1a,c,e) are named according to the terminology employed by BAILEY *et al.* (1970) for the king salmon.

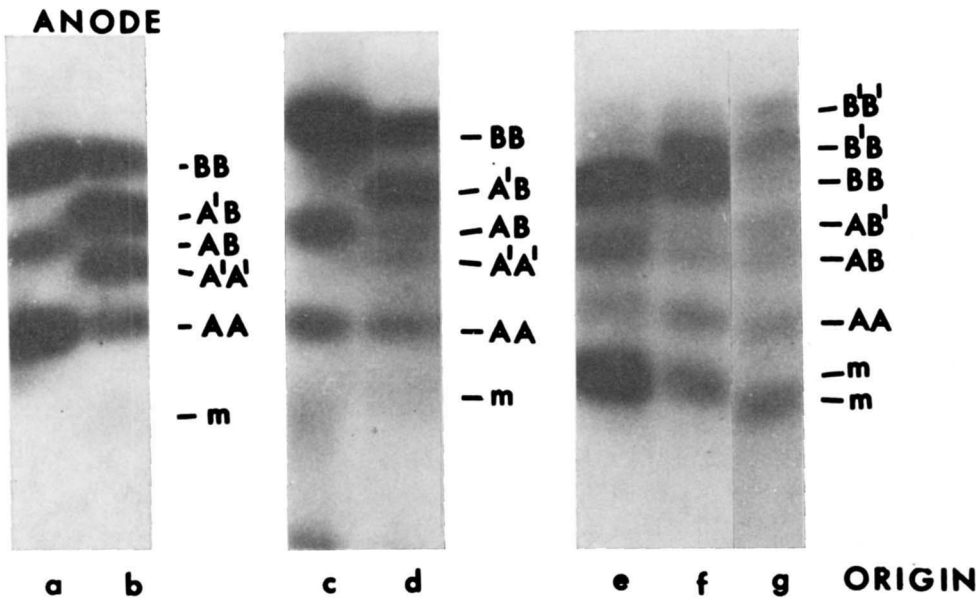


FIGURE 1.—MDH phenotypes of the pink salmon, *Oncorhynchus gorbuscha*. Muscle samples. Electrophoresis of a,b,c, and d conducted at pH 6.0; electrophoresis of e,f, and g conducted at pH 6.8. All possible phenotypes are not represented here. m = mitochondrial MDH.

They are: AA, AB, BB. These isozymes are produced by the association of A and B subunits. In passing, mention should be made of one discrepancy noted between this study and the one of BAILEY *et al.* (1970). They found that B-type subunits were the predominant ones in king salmon skeletal muscle. A-type subunits were apparently absent in isozymes from skeletal muscle judging from the zymograms presented in their paper. In contrast, subunit A was detected in skeletal muscle of the pink salmon (Figure 1) although usually not as abundant as the B-type subunit.

Less common phenotypes were found which possessed additional isozymes produced by variants of subunits A and B (Figures 1b,d,f,g). Because some phenotypes possessed additional isozymes with electrophoretic mobilities more similar to the BB than to the AA isozyme (Figures 1f,g), it was assumed that these isozymes originated from a variant of subunit B, which subsequently has been named B′. Similar reasoning was applied in naming subunit A′, a variant subunit responsible for generating additional isozymes in other less common phenotypes (Figures 1b,d). However, no immunological tests were conducted to test the assumption of homology between subunits A and A′ or between subunits B and B′.

Figures 1b and 1d illustrate two phenotypes heterozygous for A and A′ subunits (subunit B also present). These two heterozygotes differ, however, in the relative concentrations of the isozymes present. The heterozygote in Figure 1b possesses relatively higher concentrations of A′B and A′A′ isozymes than the one

in Fig. 1d. Also, the ratio of the concentrations of A′B:A′A′ is much higher in the latter than in the former heterozygote. In both heterozygotes with the A′ variant, the A′A isozyme which, in theory, should have been present was detected only in excellent electrophoretic preparations. The A′A isozyme cannot be seen in the gels of Figure 1.

Figure 1f and 1g illustrate two phenotypes heterozygous for B and B′ subunits (subunit A also present). These heterozygotes differ in the relative concentrations of BB, B′B and B′B′. In the heterozygote of Figure 1f, the relative concentrations are BB>B′B>B′B′, while in the heterozygote of Figure 1g, the relative concentrations appear to be B′B>BB=B′B′.

Although not depicted in Figure 1, other phenotypes were found which were simultaneously heterozygous for A-type and B-type subunits.

*Inheritance of A-Type Subunits*

Six crosses (*5,6,7,8,10,11*) were performed in which both parents were homozygous for subunit A. All progeny from these crosses were also homozygous for subunit A (Table 1). A most revealing cross was *1* in which both parents were

TABLE 1

*Summary of genetic crosses involving MDH phenotypes of the pink salmon,* Oncorhynchus gorbuscha

| | Phenotypes of parents* | | Phenotypes of progeny* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Cross | Female | Male | A′A′AA BBBB | A′AAA BBBB | AAAA BBBB | AAAA B′BBB | AAAA B′B′BB | A′AAA B′BBB | Total |
| 1 | A′AAA BBBB | A′AAA BBBB | 25 | 54 | 23 | .. | .. | .. | 102 |
| 2 | A′AAA BBBB | AAAA B′BBB | .. | 20 | 16 | 18 | .. | 12 | 66 |
| 3 | AAAA BBBB | A′A′AA BBBB | 1 | 38 | 3 | .. | .. | .. | 42 |
| 4 | AAAA BBBB | A′AAA BBBB | .. | 49 | 40 | .. | .. | .. | 89 |
| 5 | AAAA BBBB | AAAA BBBB | .. | .. | 24 | .. | .. | .. | 24 |
| 6 | AAAA BBBB | AAAA BBBB | .. | .. | 15 | .. | .. | .. | 15 |
| 7 | AAAA BBBB | AAAA B′BBB | .. | .. | 15 | 15 | .. | .. | 30 |
| 8 | AAAA BBBB | AAAA B′BBB | .. | .. | 32 | 38 | .. | .. | 70 |
| 9 | AAAA B′BBB | A′A′AA BBBB | .. | 4 | 1 | .. | .. | 5 | 10 |
| 10 | AAAA B′BBB | AAAA BBBB | .. | .. | 21 | 28 | .. | .. | 49 |
| 11 | AAAA B′BBB | AAAA B′BBB | .. | .. | 7 | 10 | 5 | .. | 22 |

* Phenotypes are expressed as the relative numbers of A and A′ subunits and B and B′ subunits assuming, according to the genetic model presented in this paper, each individual possesses a total of four A-type and four B-type subunits (alleles).

heterozygous (phenotype of Figure 1d) for A and A′ subunits. This cross produced progeny in a ratio of approximately 3:1 ($\chi^2 = 0.32$, P>.50) with 79 individuals heterozygous for subunits A and A′ and 23 individuals homozygous for A (Table 1). Furthermore, the heterozygous progeny were discernible as two classes with approximately one-third displaying the heterozygous phenotype of Figure 1b and two-thirds the heterozygous phenotype of Figure 1d. Surprisingly, no progeny lacked the A subunit. It was expected in a heterozygous × heterozygous mating that some progeny would be homozygous for the A′ subunit. That this did not occur supports the hypothesis that the A-type subunit is encoded by two loci with four alleles. Under this hypothesis then, the two classes of heterozygotes discernible in cross *1* either have one or two doses of the A′ subunit and are designated accordingly as the A′AAA (Figure 1d) and A′A′AA (Figure 1b) phenotypes, respectively. Studies of the relative concentrations of various isozymes in these two heterozygous phenotypes support this contention. Assuming a completely random association of subunits produced in equal quantities, the A′A′ isozyme should exist at four times the concentration in the A′A′AA than in the A′AAA phenotype. Although densimetric values were not determined, it was obvious that the concentration of the A′A′ isozyme was at its highest in the A′A′AA phenotype.

Additional evidence that the heterozygous phenotype of Figure 1b and 1d are correctly interpreted as A′A′AA and A′AAA, respectively, was obtained from studies of natural populations in 1969 and 1971. The MDH phenotype of 1,484 pink salmon was determined. Under the assumptions of the Hardy-Weinberg equilibrium, one would expect a relatively rare allele (A′ in this case) to occur much more frequently in individuals in a single dose than in a double one. The phenotype which was interpreted as A′AAA (Figure 1d) was 35 times more abundant than the one interpreted as A′A′AA (Figure 1b). These field studies provided some evidence, also, for the existence of two loci encoding for A-type subunits. Although 108 individuals possessed the A′ variant, none ever lacked the A subunit.

Crosses *3* and *9* were matings of an A′A′AA male and AAAA females. In both crosses the majority of the progeny was of the A′AAA phenotype. Surprisingly, a few progeny were A′A′AA or AAAA. The significance of this will be discussed later.

*Inheritance of B-Type Subunits*

Five crosses (*1*,*3*,*4*,*5*,*6*) were conducted with both parents homozygous for subunit B. As anticipated, all progeny were homozygous for subunit B (Table 1). Cross *11* was a mating of pink salmon both of whom were heterozygous (phenotype of Figure 1f) for subunits B and B′. The progeny conformed to a 3:1 ratio ($\chi^2 = 1.3$, P>.25) with 15 individuals heterozygous for subunits B and B′ and 7 individuals homozygous for the B subunit. The heterozygous individuals were discernible into two distinct classes with two-thirds of them displaying the heterozygous pattern of Figure 1f and one-third the heterozygous pattern of Figure 1g. None of the progeny in this cross were observed in which subunit B′ was homozy-

gous, although this type would be expected from a heterozygote (B′B) × heterozygote (B′B) cross. This observation provides strong evidence to support the assertion of duplicate loci encoding for B-type subunits in pink salmon. According to this hypothesis, each individual possesses four alleles encoding for B-type subunits; the phenotype in Figure 1f can be interpreted as a B′BBB heterozygote and the one in Figure 1g as a B′B′BB heterozygote. The relative concentrations of BB, B′B, and B′B′ isozymes in the two heterozygous phenotypes are also consistent with the two-loci hypothesis. In the B′BBB heterozygote, random assembly of B and B′ subunits synthesized in a ratio of 3:1 should produce BB, B′B, and B′B′ isozymes in a ratio of 9:6:1, whereas the respective ratios should be 1:2:1 in the B′B′BB heterozygote, assuming equal quantities of B and B′ subunits. The observed ratios of the isozymes in the two heterozygous phenotypes appear to be consistent with the expected ratios.

The study of natural populations of pink salmon reported previously in this paper also supported the hypothesis of duplicate loci encoding for B-type subunits. One hundred and thirty-eight adults were observed which possessed the B′ subunit. All of these individuals, however, possessed the B subunit as well.

## DISCUSSION

The investigations of BAILEY *et al.* (1970) have demonstrated the presence of duplicate loci encoding for B-type subunits in the king salmon. Also, evidence from isozyme dosage studies indicates the duplication of loci encoding A-type subunits in the brown trout. Because neither the king salmon nor the brown trout possesses polymorphisms at loci encoding for both A-type and B-type subunits, it was not possible to demonstrate the duplication of the two loci in either species. The presence of duplicate loci encoding for both A- and B-type subunits was established for the pink salmon because of the existence of polymorphisms for both types of loci. This is the first such finding, to my knowledge, for s-MDH in salmonids.

The presence of duplicate loci, along the with DNA measurements and chromosome studies, has been cited as evidence for the tetraploid nature of this family (OHNO 1970). However, the presence of duplicate loci and/or large quantities of DNA is circumstantial evidence for the existence of polyploidy. Tandem duplication of genes, or clusters of genes, could produce similar results as appears to be the case in the evolution of the lungfish, *Lepidosiren* (OHNO 1970). Evidence in the present study suggests that the duplicate loci encoding for A-type subunits may be, in fact, linked. Two crosses (*3* and *9*) of A′A′AA × AAAA yielded, collectively, 47 A′AAA, 4 AAAA and 1 A′A′AA progeny. Several models were constructed to explain the unexpected results of these particular crosses (Table 2). A model consistent with the observed results is model 1 which assumes that both loci encoding for A-type subunits are polymorphic and linked (in repulsion phase in these particular crosses). The high ratio of A′AAA to AAAA and A′A′AA (10.4:1.0) suggests a crossing-over event.

Model 3, which assumes two polymorphic unlinked loci and a high frequency of centric fusions between chromosomes bearing A and A′ alleles, is also con-

TABLE 2

*Genetic models for the control of loci encoding for A-type subunits (based on data from crosses 3 and 9)*

| Model | Proposed genotypes of parents based on phenotypes of crosses 3 and 9 — Phenotypes A'A'AA♂ | AAAA♀ | Expected results (phenotypes of progeny) |
|---|---|---|---|
| | repulsion phase | | |
| | A' A | A A | ?% crossover progeny, AAAA |
| | × | | ?% non-crossover progeny, A'AAA |
| | A A' | A A | ?% crossover progeny, A'A'AA |
| 1. Both loci polymorphic and linked | coupling phase | | |
| | A' A' | A A | ?% non-crossover progeny, AAAA |
| | × | | ?% crossover progeny, A'AAA |
| | A A | A A | ?% non-crossover progeny, A'A'AA |
| 2. Both loci polymorphic and unlinked | A' A' | A A | 25% of progeny, AAAA |
| | × | | 50% of progeny, A'AAA |
| | A A | A A | 25% of progeny, A'A'AA |
| 3. Both loci polymorphic and unlinked | A' A' | A A | |
| | × | | |
| | A A | A A | Large % of progeny, A'AAA |
| and | | | Small % of progeny, A'A'AA |
| high frequency of centric fusions | A' A | A A | Small % of progeny, AAAA |
| | × | | |
| | A A' | A A | |
| 4. Single tetrasomic locus with random association of homologues during meiosis | A' | A | 25% of progeny, AAAA |
| | A' | A | 50% of progeny, A'AAA |
| | × | | |
| | A | A | 25% of progeny, A'A'AA |
| | A | A | |

sistent with the data and was suggested by the investigations of MORRISON (1970) and of DAVISSON, WRIGHT and ATHERTON (1973). These workers discovered, sometimes, a high degree of nonrandom assortment of alleles between LDH A and B loci in male brook trout and in male splake (lake trout × brook trout hybrids). However, the alleles of these loci always assort independently in females, demonstrating that true linkage is not involved. This phenomenon, termed pseudolinkage, is apparently caused by centric fusion of two acrocentric chromosomes, one bearing the LDH A locus and the other the LDH B locus. Pseudolinkage, which can be as high as 99% in a particular cross (MORRISON 1970), can break down by a corresponding fission process (DAVISSON, WRIGHT and ATHERTON 1973). In crosses 3 and 9, both of which involved the same male of phenotype A'A'AA, the high proportion (90%) of A'AAA progeny could have resulted from selective centric fusions of chromosomes carrying the A and A' allele.

Model 2, also a non-linkage model, is inconsistent with these data since the

expected ratios of AAAA:A'AAA:A'A'AA should be 1:2:1, respectively. Tetrasomic inheritance with random association of chromosomal homologs during anaphase (model 4), likewise, is inconsistent with these data since the ratio of AAAA:A'AAA:A'A'AA should also be 1:2:1. If the homologs of a tetrasomic locus should selectively associate during meiosis on the basis of genetic similarity (with respect to MDH), A'A'AA and AAAA phenotypes should have predominated. This is exactly opposite of what was found in crosses *3* and *9*.

Clearly, further experiments are needed to confirm which model of the four, if any, is correct. What is needed are the results of numerous reciprocal matings of A'A'AA × AAAA. Pseudolinkage could be established if A'A'AA females assort independently, as is the case in female splake and brook trout. The confirmation of pseudolinkage of MDH-A loci, rather than true linkage, would lend support to the contention that salmonids are tetraploids.

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