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III. Linkage between two duplicated loci coding for aspartate aminotransferase in the cutthroat trout (*Salmo clarki*)

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The genetic control of the supernatant form of aspartate aminotransferase (AAT) was studied in the cutthroat trout (*Salmo clarki*) through a series of experimental matings. 509 individuals of eight families were examined to determine (1) the number of loci, (2) the mode of inheritance (i.e. disomic or tetrasomic), and (3) the linkage relationship of the loci involved. The variation observed is controlled by a duplicated locus resulting from a presumed tetraploid event of an ancestral salmonid. The inheritance experiments revealed the presence of two disomic loci rather than a single tetrasomic locus, indicating that disomy has been reestablished for the chromosomes carrying the AAT loci. The two families in which linkage between these loci could be tested displayed significant nonrandom segregation between these loci with an estimated frequency of recombination of 30.6%. These results are discussed in regard to the proposed evolution of tetraploidy in the family Salmonidae.

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Tetraploidy is an important evolutionary occurrence which may have been responsible for large increases in genome size and diversity early in vertebrate evolution (OHNO 1970). There is a growing body of evidence that an ancestor of present day mammals went through at least one tetraploid event which is responsible for much of the gene duplication seen in vertebrates today (COMINGS 1972; OHNO 1973).

Isozymic studies have revealed that the fishes of the family Salmonidae possess a great deal of gene duplication in comparison to other vertebrates (BAILEY and WILSON 1968; BAILEY et al. 1970; ALLENDORF et al. 1975). OHNO has proposed that the salmonid lineage has gone through an additional tetraploid event on the basis of this gene multiplicity, comparative amounts of DNA, and certain chromosomal characteristics (OHNO 1974). Because of this additional gene duplication the salmonids represent an excellent opportunity to study the evolution of duplicated genes in a group of vertebrate species.

Previous papers in this series have been concerned with the genetic analysis of duplicated loci (ALLENDORF and UTTER 1973; ALLENDORF et al. 1975). The present communication deals with the genetic analysis of duplicated loci coding for the supernatant form of the enzyme aspartate aminotransferase (AAT; glutamic-oxalacetic transaminase; E.C. 2.6.1.1) in the cutthroat trout (*Salmo clarki*). Examination of populations of this species for AAT revealed a polymorphic system which is best explained by the presence of a duplicated locus. The purpose of this paper is to outline the genetic control of this enzyme in cutthroat trout; this analysis includes (1) the determination of the number of loci coding for this enzyme, (2) the mode of inheritance, and (3) the linkage relationship of the loci involved.

Materials and methods

Electrophoresis

Muscle extracts were prepared and horizontal starch gel electrophoresis was accomplished following the methods of UTTER et al. (1974). Gels were made using 14% starch in a buffer consisting of 99% tris (0.03 M) and citric acid (0.005 M) and 1% of the

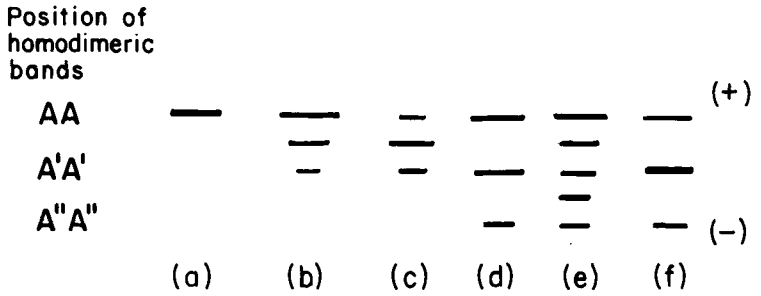

Fig. 1. Diagrammatic representation of six of the possible phenotypes observed for supernatant AAT in cutthroat trout. The phenotypes seen above are (a) AAAA, (b) AAAA′, (c) AAA′A′, (d) AAAA″, (e) AAA′A″, and (f) AAA″A″. Additional phenotypes which are not shown are those in which an individual has more than two doses of a variant allele (e.g. AA′A′A′).

bridge buffer. The bridge buffer consisted of lithium hydroxide (0.06 M) and boric acid (0.03 M) (RIDGWAY et al. 1970). A potential of 200 V was applied across the gel until the borate boundary was 6 cm from the origin. Staining for specific AAT activity was done following the methods of JOHNSON et al. (1972).

Matings

Sexually mature anadromous cutthroat trout were obtained from the Cowlitz Hatchery of the Washington State Game Department and the intact carcasses transported on ice to the laboratory. The gametes were then removed, placed in individual plastic bags, and stored at 5°C overnight. Muscle tissue samples were taken from these fish at the same time the gametes were collected. These samples were tested electrophoretically and selected matings made the following day on the basis of these results. Progeny testing was initiated when the progeny reached a length of approximately 3 cm.

Results

Phenotypes

Previous studies have shown the supernatant form of AAT in vertebrates to be anodally migrating at a neutral pH (in contrast to the migration of the mitochondrial form which is cathodal) and to have a dimeric structure (DE LORENZO and RUDDLE 1970). The supernatant form of AAT has been found to be coded for by a single locus in most vertebrate species but has been shown to be coded for by two loci in several fish species presumed to be of recent tetraploid origin (SCHMIDTKE and ENGEL 1972). Two disomic loci have been reported to code for AAT in both the brown trout (*Salmo trutta*) (SCHMIDTKE and ENGEL 1972) and the chum salmon (*Oncorhynchus nerka*) (ALLENDORF et al. 1975). Inheritance studies have shown these loci to be unlinked in the chum salmon (ALLENDORF et al. 1975).

Five different electrophoretic phenotypes from muscle extracts were observed in the cutthroat trout population used for the experimental matings (Fig. 1). The five-banded phenotype seen for this dimeric enzyme is explained by the presence of three different alleles in a single individual and is therefore evidence of a duplicated locus coding for this enzyme. In addition, the asymmetric phenotypes seen (Fig. 1b, d) are typical of those described for duplicated loci where a simple heterozygote has a single dose of the variant allele and three doses of the common allele (ALLENDORF et al. 1975). On the basis of these results, it was concluded that AAT in cutthroat trout is coded for by a duplicated locus.

These multiple-banded phenotypes are explained by the presence of three electrophoretically distinct alleles. The most frequent variant allele (AAT-A′) has a mobility relative to the common allele (AAT-A) of (0.88); the second variant allele (AAT-A″) has a relative mobility of (0.77).

Inheritance experiments

Forty sexually mature cutthroat trout were examined and 15 matings made on the basis of the parental genotypes for AAT as well as other enzymes not reported here. The rationale of the method used to select the matings to determine the control of these

Table 1. Observed and expected segregation of AAT alleles in cutthroat trout

Lot no.	Parental phenotypes (Presumed genotypes)		Progeny phenotypes: observed (expected with disomic inheritance) /expected with tetrasomic inheritance/							χ^2	df	P
	Female	Male	AAAA	AAAA′	AAA′A′	AA′A′A′	A′A′A′A′	AAAA″	AAA′A″			
C1	AAAA	AAA′A″	53	30				21	65			
	($A_1A_1A_2A_2$)	($A_1A_1'A_2A_2''$)	(42.3)	(42.3)				(42.3)	(42.3)	29.2	3	<.001
			/28.2/	/56.3/				/56.3/	/28.2/	104.2	3	<.001
C4	AAA′A′	AAAA		40								
	($A_1A_1A_2'A_2'$)	($A_1A_1A_2A_2$)		(40.0)						0	0	
			/6.7/	/26.7/	/6.7/					20.0	2	<.001
C5	AAA′A′	AAAA	30	30	38							
	($A_1A_1'A_2A_2'$)	($A_1A_1A_2A_2$)	(24.5)	(49.0)	(24.5)					16.0	2	<.001
			/16.3/	/65.3/	/16.3/					59.5	2	<.001
C8	AAA′A′	AAAA		35								
	($A_1A_1A_2'A_2'$)	($A_1A_1A_2A_2$)		(35.0)						0	0	
			/5.8/	/23.3/	/5.8/					17.5	2	<.001
C9	AAA′A′	AAAA		38								
	($A_1A_1A_2'A_2'$)	($A_1A_1A_2A_2$)		(38.0)						0	0	
			/6.3/	/25.3/	/6.3/					19.0	2	<.001
C12	AA′A′A′	AAAA		19	25							
	($A_1A_1'A_2'A_2'$)	($A_1A_1A_2A_2$)		(22.0)	(22.0)					0.8	1	>.50
				/22.0/	/22.0/					0.8	1	>.50
C14	AAAA	AAA′A″		12				12				
	($A_1A_1A_2A_2$)	($A_1A_1A_2'A_2''$)		(12.0)				(12.0)		0	1	
			/4.0/	/8.0/				/8.0/	/4.0/	12.0	3	<.010
C15	AAA′A′	AAA′A′	2	14	24	18	3					
	($A_1A_1'A_2A_2'$)	($A_1A_1'A_2A_2'$)	(3.8)	(15.3)	(22.9)	(15.3)	(3.8)			1.7	4	>.90
			/1.7/	/13.6/	/30.5/	/13.6/	/1.7/			4.4	4	>.50

Table 2. Test for linkage between duplicated AAT loci in cutthroat trout

Parental genotypes		Progeny phenotypes					Test for 1:1 segregation within both loci (2 df)		Test for linkage (1 df)	
Female	Male	AAAA	AAAA′	AAA′A′	AAAA″	AAA′A″	χ^2	P	χ^2	P
$A_1A_1A_2A_2$	$A_1A_1'A_2A_2'$	53 (42.2)	30 (42.2)		21 (42.2)	65 (42.2)	2.62	> .25	26.56	< .0001
$A_1A_1A_2A_2$	$A_1A_1'A_2A_2'$	30 (23.5)	29 (47.0)	35 (23.5)					13.79	< .001
Sum (2 df)									40.35	< .0001

duplicated loci has been described (ALLENDORF et al. 1975). Selected families from these matings were examined to demonstrate the genetic control of AAT; the data obtained are presented in Table 1. The presumed genotype of the parents listed in this table was determined on the basis of the observed segregation in the progeny from these individuals. The designations A_1 and A_2 refer only to alleles at distinct loci within a single individual and can not be compared between individuals.

The presence of two disomic loci rather than a single tetrasomic locus is demonstrated by the results from families C4, C8, and C9. The parental phenotype AAA′A′ can represent any one of three possible genotypes:

(1) $A_1A_1A_2'A_2'$ (disomic)
(2) $A_1A_1'A_2A_2'$ (disomic)
(3) AAA′A′ (tetrasomic)

The expected gamete segregation ratios from each of these genotypes are different. The matings of individuals of this phenotype to single-banded homozygotes provides a test between the two models of inheritance (i.e. disomy or tetrasomy). In the families C4, C8, and C9 only a single phenotype (AAAA′) was seen in the progeny, confirming that the multiple-banded parent in each case was of genotype (1) above. In the case of tetrasomic inheritance, the alleles are expected to segregate as discussed below, providing at least three phenotypic classes in the progeny.

The actual observed tetrasomic segregation ratio from the genotype AAA′A′ will vary depending on the genetic distance between the locus in question and its centromere. If the locus is very close to the centromere, the observed segregation should closely approximate a ratio of 1:4:1 (AA:AA′:A′A′) (chromosome segregation). If, however, the locus is distant enough from the centromere to allow free recombination, then the observed segregation will approximate a ratio of 3:8:3 (chromatid segregation). This ratio is very close to the 4:8:4 ratio expected with unlinked disomic loci of the parental genotype $A_1A_1'A_2A_2'$. An added complication is the possible linkage of the two disomic loci. Depending on the intensity of the linkage, the segregation ratio of the genotype $A_1A_1'A_2A_2'$ can vary between 0:1:0 or 1:0:1 (depending on the linkage phase) to the 1:2:1 expected for unlinked loci. The conclusion from this analysis is that to conclusively demonstrate the existence of disomic inheritance in a population, both the double homozygote ($A_1A_1A_2'A_2'$) and the double heterozygote ($A_1A_1'A_2A_2'$) parental types must be found in the population to avoid any confounding problems caused by linkage. This criterion is met in the present

data; double heterozygous parental types are seen in families C1, C5, and C15 while double homozygous parental types occur in families C4, C8, C9, and C14.

The frequency of recombination between these two loci can be estimated by the progeny results from families C1 and C5 (Table 2). The double heterozygous parent of family C1 possesses different variant alleles at the two loci, allowing all four progeny classes normally seen in a linkage test-cross to be identified. In this family, both the segregation of complementary gametes within each locus and the joint segregation between loci can be tested. The chi-square values for the within locus segregation of both loci are well within the conventional 95% interval. However, the chi-square value for independent assortment of these loci has a probability less than 0.01% ($\chi^2 = 26.6$; df = 1). The estimated frequency of recombination between loci in this family is 30.2%.

Family C5 could not be tested for the equality of complementary gametes within loci because the genotypes $A_1A_1'A_2A_2$ and $A_1A_1A_2A_2'$ can not be distinguished in the progeny. However, since these two genotypes comprise one of the two linkage classes, this family can be used to test the frequency of recombination between loci. The chi-square value for independent assortment between loci for this family has a probability of less than 0.1% ($\chi^2 = 13.8$; df = 1) with an estimated frequency of recombination of 30.9%. Pooling these two families yields a chi-square value for independent assortment between the loci of 40.4 (df = 2; probability less than 0.01%) and an average frequency of recombination of 30.6%.

Discussion

The observation of quadrivalents in meiosis of some salmonid species has led to the prediction that some loci in salmonids should be found to segregate tetrasomically (OHNO et al. 1968). There is presently, however, no evidence of tetrasomic inheritance in any present-day salmonid; every locus whose inheritance has been reported has been found to segregate disomically (DAVISSON et al. 1973; ENGEL et al. 1975; ALLENDORF et al. 1975). Therefore, sufficient time has elapsed since the presumed tetraploid event early in salmonid evolution to allow the reestablishment of disomy in at least the great majority of loci.

How has this disomy evolved? OHNO has proposed that the establishment of chromosomes which are the products of a centric fusion between the homologous duplicated acro- or telo-centric chromosomes may be one mechanism whereby disomy is reestablished (OHNO et al. 1970). Preferential fusion of the chromosomes carrying the two loci resulting from the duplication of the vertebrate lactate dehydrogenase A locus (LDH-A) has been thoroughly described by both isozymic and cytogenetic analysis of hybrids between lake trout (*Salvelinus namaycush*) and brook trout (*Salvelinus fontinalis*) (DAVISSON et al. 1973; MORRISON 1970). A similar phenomenon for these same loci was also reported to occur in hybrids between two different strains of rainbow trout (*Salmo gairdneri*) (WRIGHT et al. 1975). The establishment of such fusions in a species would result in the disomic inheritance of both loci within a pair and would also place both loci in the same linkage group. On the basis of the inheritance data reported here, such a fusion appears to have occurred between the chromosomes containing the duplicated AAT loci in cutthroat trout.

Of special interest is a recent study of the evolutionary divergence of the same two duplicated LDH-A loci which have been shown to be on chromosomes which undergo selective fusion (LIM et al. 1975). On the basis of comparative quantitative immunological experiments, it was concluded that these two loci have been genetically distinct for approximately 100 million years, and that the duplication event producing these loci occurred at that time. This latter conclusion is a conservative estimate, however. Following tetraploidy, the divergence of the two loci resulting from the duplication of a single locus can not begin until disomic inheritance has been established for these loci. This principle is demonstrated by the many pairs of duplicated loci in the salmonids for which there is no evidence of evolutionary divergence between the two loci in a duplicated pair (e.g. AAT in cutthroat trout). The absence of detectable divergence within these pairs of duplicated loci indicates that the disomic inheritance of these loci has probably been established in the comparatively recent past. The length of time that the two LDH-A loci have been distinct therefore represents a minimum estimate of the time since the duplication event. The important observation, however, is that these two pairs of homeologous chromosomes still retain sufficient homology to undergo a process of selective fusion an estimated 100 million years after their separation.

There now are several reports of non-random joint segregation between loci within duplicated pairs of genes in the salmonids. As well as the linkage described in this paper and the duplicated LDH loci discussed previously, ASPINWALL (1974) has reported limited evidence of non-random joint segregation of

two malate dehydrogenase loci in the pink salmon (*Oncorhynchus gorbuscha*). Further investigations of the linkage relationships of these and other duplicated loci pairs are critical for understanding the evolution of duplicated loci in the salmonids. The continuing investigation of the genetic and biochemical relationships of duplicated gene pairs in the salmonids will further contribute to our understanding of the role of gene duplication in the evolution of the vertebrate genome.

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