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Electrophoretic Analysis of *Campostoma anomalum, Rhinichthys cataractae* and their F_1 Offspring

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Key Word Index – *Campostoma anomalum*; *Rhinichthys cataractae*; Cyprinidae; Cypriniformes; isozymes; acrylamide electrophoresis; hybrid; muscle and serum proteins.

Abstract – *Campostoma anomalum, Rhinichthys cataractae* and their F_1 hybrids were examined electrophoretically for 44 enzymatic loci, general muscle, and serum proteins. Of the 44 loci scored, acid phosphatase (ACP-B), alkaline phosphatase (AKP-A), esterase (EST-B), α-glycerophosphate dehydrogenase (GPD-A), malate dehydrogenase (MDH-A) and phosphoglucomutase (PGM-A) showed hybrid inheritance patterns. Serum proteins also demonstrated additive inheritance patterns, comprising 15 serum proteins in the parents and 18 in the F_1 hybrid. Banding patterns for all mixtures of parental species were identical to those observed in the hybrid.

Introduction

Both meristic and biochemical analyses may be used to confirm hybridization in fishes. Hubbs [1] in his erudite discussion of hybridization commented on the rule that natural interspecific hybrids demonstrate intermediate meristic characters between their parental species. This intermediacy may range over characters as diverse as shape, color, structure, or number of anatomical features. Recognizing hybrids becomes increasingly difficult as morphological similarities between the parents increases [2]. When hybrids are morphologically quite similar, this may lead to misidentification of hybrids and/or their parents. Morphological intermediacy does not necessarily indicate hybrid origin; one species may be intermediate to two others merely as a result of stochastic selection [2].

Biochemical characters for hybrids also tend to show intermediate inheritance patterns for protein systems distinct for the parental species [2]. This additive effect occurs when homologous proteins from the two parental species differ electrophoretically. When this occurs the hybrid usually inherits both isozymes from the parents via simple Mendelian inheritance, thus becoming heterozygous.

Intrageneric and intergeneric hybrids are well known in the freshwater fish family Cyprinidae, including the genera *Campostoma* and *Rhinichthys* [3]. Primarily found in streams with moderate to swift currents over gravel substrates, *Campostoma anomalum* (stoneroller) and *Rhinichthys cataractae* (longnose dace) hybridize naturally [3–6]. Schwartz [3] listed 10 hybrid cases between *Campostoma anomalum* and other cyprinids and 11 examples of *Rhinichthys cataractae* hybridization. Many cyprinid hybrids occur from concurrent spawning activity of parental species over *Nocomis* nests [1, 7, 8]. For a discussion of hybridization in fishes, see Hubbs [1] and Stauffer *et al.* [9].

This paper describes biochemical analyses of *Campostoma anomalum, Rhinichthys cataractae* and their hybrid offspring from the Cheat River drainage of West Virginia.

Results

Each specimen (37 individuals) was analysed for gene products of 44 loci resolving 86 enzymatic alleles (Table 1). A total of four soluble muscle proteins was consistently observed on isoelectric focusing gels for the stoneroller, longnose dace and their hybrid. On gradient slab acrylamide gels, all muscle proteins were identical among all of the samples. Fifteen serum proteins were distinctly observed for both stoneroller and longnose dace, but 18 serum proteins appeared in electropherograms (gradient slab acrylamide) of the hybrid (Fig. 1).

Of the 44 enzyme loci scored, six (ACP-B, AKP-A, EST-B, GPDH-A, MDH-A and PGM-A) showed hybrid inheritance patterns (Fig. 2). These

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TABLE 1. ENZYME SYSTEMS EXAMINED

Enzyme	EC number	Locus	No. of alleles resolved	Ref. to stain system
Acid phosphatase	3.1.3.2	ACP-A	2	[31]
		ACP-B	3	
		ACP-C	1	
Adenylate kinase	2.7.4.3	ADK-A	1	[31]
		ADK-B	1	
Alanine dehydrogenase	1.4.1.1	ALDH-A	3	[29]
Aldolase	4.1.2.13	ALD-A	2	[29]
Alkaline phosphatase	3.1.3.1	AKP-A	2	[31]
Alcohol dehydrogenase	1.1.1.1	ADH-A	2	[33]
Catalase	1.11.1.6	CAT-A	2	[31]
Creatine kinase	2.7.3.2	CK-A	2	[33]
		CK-B	1	
Esterase	3.1.1.1	EST-A	2	[32]
		EST-B	3	
Fructose 1,6-diphosphate dehydrogenase	3.1.3.11	FDD-A	2	[32]
Fumerase	4.2.1.2	FUM-A	2	[32]
Glucose-6-phosphate *dehydrogenase*	1.1.1.49	G6PHD-A	1	[31]
		G6PHD-B	2	
Glucose phosphate isomerase	5.3.1.9	GPI-A	3	[33]
Glutamate dehydrogenase	2.6.1.2	GDH-A	1	[31]
		GDH-B	3	
Glutamate-oxaloacetate transaminase	2.6.1.1	GOT-A	1	[31]
		GOT-B	2	
α-Glycerophosphate dehydrogenase	1.1.1.8	GPD-A	2	[30]
		GPD-B	3	
Hexokinase	2.7.1.1	HK-A	1	[31]
Lactate dehydrogenase	1.1.1.27	LDH-A	1	[30]
		LDH-B	1	
Leucine aminopeptidase	3.4.1.1	LAP-A	2	[31]
Malate dehydrogenase	1.1.1.37	MDH-A	2	[33]
		MDH-B	3	
Malic enzyme	1.1.1.40	ME-A	1	[31]
Mannose-6-phosphate isomerase	5.3.1.8	MPI-A	1	[31]
Nucleoside phosphoralase	2.4.2.1	NSP-A	1	[31]
Octanol dehydrogenase	1.1.1.2	ODH-A	1	[31]
		ODH-B	3	
Phosphoglucomutase	2.7.5.1	PGM-A	2	[33]
6-Phosphogluconate dehydrogenase	1.1.1.44	6-PGD-A	2	[31]
Pyruvate kinase	2.7.1.40	PK-A	2	[31]
Sorbitol dehydrogenase	1.1.1.14	SoDH-A	3	[31]
Superoxide dismutase	1.15.1.1	SOD-A	1	[33]
		SOD-B	2	
Xanthine dehydrogenase	1.2.1.37	XDH-A	2	[31]
		XDH-B	3	

inheritance patterns were always additive (and, thus, intermediate) in that if the two parental species had distinct alleles occurring at a locus, then the hybrid expressed both alleles (Fig. 2). In all cases, the hybrid pattern was identical to the enzyme pattern obtained by mixing samples from the two parental species.

Serum protein patterns of the stoneroller, longnose dace and their hybrid were separated into

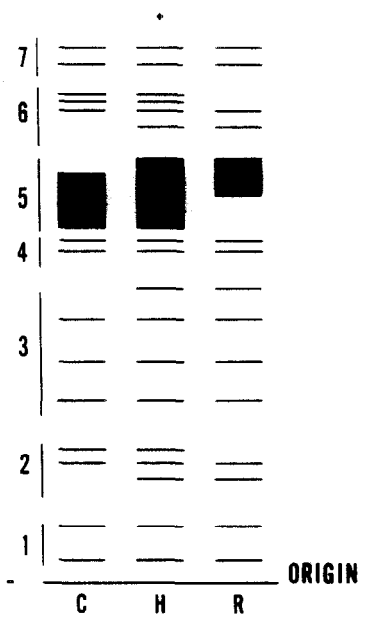

FIG. 1. SERUM PROTEINS OF *CAMPOSTOMA ANOMALUM* (C), *RHINICHTHYS CATARACTAE* (R) AND THEIR HYBRID (H).

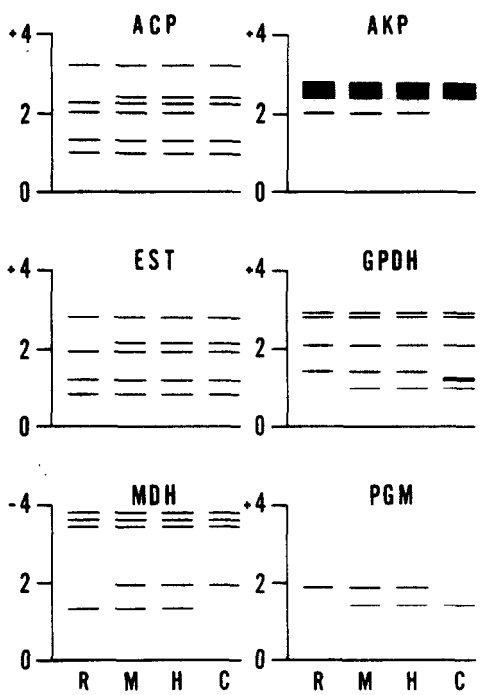

FIG. 2. ELECTROPHORETIC MOBILITIES OF ENZYMES AMONG *RHINICHTHYS CATARACTAE* (R), MIXTURE (M) OF *RHINICHTHYS CATARACTAE* AND *CAMPOSTOMA ANOMALUM* AND THE HYBRID (H) OF *CAMPOSTOMA ANOMALUM* (C).

seven distinct and easily scorable regions (Fig. 1). No attempt was made to assign the occurrence of a serum protein with a presumed loci, rather regions of similar electrophoretic mobility and density were examined. Many minor bands were also observed, but scoring was difficult for these serum proteins. Of the seven regions (Fig. 1), additive inheritance patterns were observed in regions 2, 3, 5 and 6. In region 5 (Fig. 1), the additive pattern appeared as a wider and denser serum protein.

Discussion

The hybrid between *Campostoma anomalum* and *Rhinichthys cataractae* showed molecular inheritance patterns for 6 of 44 enzymatic loci, 0 of 4 muscle proteins, and 4 of 7 serum protein regions consisting of 15 serum proteins for both parents and 18 for the hybrid. The hybrid is most readily identified by the array of serum proteins in regions 2 and 5 (Fig. 1). Four enzymes, ACP, EST, MDH and PGM, most clearly distinguish the hybrid from the parental species. Banding patterns for all mixtures of parental proteins were identical to those observed in the hybrid.

For freshwater fishes, similar results were obtained for centrarchid hybrids [10–12], which exhibited similar biochemical intermediacy as obtained in this study. In the Percidae, additive effects in muscle myogen patterns between johnny (*Etheostoma nigrum*) and tessellated (*E. olmstedi*) darters facilitated identification of hybrids [13]. Electrophoretic separation of MDH [14] discriminated walleye (*Stizostedion vitreum*) × sauger (*S. canadense*) hybrids from their parents. Salmonid hybrids [15–17] have also demonstrated additive inheritance patterns electrophoretically.

Similar results have also been reported in other cyprinid crosses, such as *Richardsonius balteatus* × *Mylocheilus caurinum* [2], *Notropis cerasinus* × *Notropis cornutus* [18] and hybrids of *Abramis brama, Rutilus rutilis* and *Scardinus erythrophthalmus* [19, 20]. Inheritance patterns of LDH between *Rhinichthys atratulus* and *R. cataractae* were used to readily identify the laboratory produced hybrid by Clayton and Gee [21].

Natural hybrids may demonstrate both phenotypic and genotypic intermediacy. Hubbs [1] reported that the vast majority of hybrids possess character indices whose values are intermediate between those of their parental forms. Characters of the hybrid may also lie outside the parental forms. This phenomenon, termed luxuriance, is brought about by complementary action of genes found in the parental species [22–24]. Reish *et al.* (unpublished data), using principal component analysis, discriminate analysis and a hybrid index, determined eight morphometric and meristic characters for recognizing possible hybrid *Campostoma anomalum* × *Rhinichthys cataractae*. Calculation of a hybrid index yielded a value of 54.14. The single hybrid specimen they reported on had a frenum, lacked barbels, possessed a cartilagenous lower lip, had a pharyngeal tooth count of 1, 4–4, 0, and the scales and intestine were intermediate to the parental forms. Our results support those of Reish *et al.* (unpublished data).

It appears that a common feature of molecular inheritance in hybrid freshwater fishes is the additive properties of enzymes, serum and muscle proteins. All of the work in the previously cited papers illustrated the additive nature of molecular hybridization when parental alleles were distinct. Indeed, addition facilitated the identification of suspected hybridization in several cases.

Isozyme variability of both parents, *Campostoma anomalum* [25,26] and *Rhinichthys cataractae* [27], has been investigated. *C. anomalum* was found by Buth and Burr [25] to have a mean heterozygosity ($\bar{H}$) of 6.3% of its loci and 19.7% of the loci were polymorphic in eight populations. Zimmerman *et al.* [26] agreed with Buth and Burr [25] finding a $\bar{H}$ of 6.4% of its loci, but found a significantly higher polymorphic value of 35% for populations. Both investigators found that heterozygosity values had a wide range of variability for different populations. Merritt *et al.* [27] reported that *R. cataractae* had a slightly lower $\bar{H}$ of 5.4% of its loci and 15% polymorphism for 13 populations. The Connecticut River system's heterozygosity was found to vary with latitude, with the northern populations having the higher values [27].

C. anomalum and *R. cataractae* are widespread throughout much of North America, with the latter having a slightly larger range (see Lee *et al.* [28]). Overlap of ranges, similar habitat requirements, and concurrent spawning over *Nocomis* nests [8] may be responsible for the hybridization of these species. It is concluded from electrophoretic information that hybrids collected are indeed *Campostoma anomalum* × *Rhinichthys cataractae*. In all cases hybrid banding patterns were identified to the mixture of both parental tissues. Review of the literature on hybridization of fishes by numerous investigators shows that electrophoretic patterns are inherited from both parents and are additive.

Experimental

Fish were collected from Horseshoe Run, Tucker County, West Virginia, along State Route 7 at distances 2.1 and 6.5

kilometers upstream from the confluence with the Cheat River. Samples were collected by herding fish downstream into a 1.5 × 3.0 m × 0.32 cm common seine with an electroshocking (220 DC) unit. Fish were removed carefully from the seine and placed into 200 l. plastic containers fitted with standard aquarium filtration–aeration systems and transported back to the laboratory, where they were held at 18°C for 5 days.

Live hybrids were identified by comparing them to existing preserved specimens located in the Fish Museum at the Appalachian Environmental Laboratory (AEL). To assist in confirmation of identification, intestinal coiling was also compared with preserved specimens after the live specimens had been killed for assay; intermediate intestine coiling occurs in this hybrid combination as compared to the herbivorous *C. anomalum* (long coiled intestine) and the omnivorous *R. cataractae* (short s-shaped intestine).

A total of 15 stonerollers, 15 longnose dace and 7 hybrids was examined electrophoretically. Muscle tissue was dissected from the two species and each hybrid. Equal parts of muscle and distilled water were homogenized using a ground glass tissue grinder; the homogenate was centrifuged at 1600 *g* for 15 min. The supernatant was used for isoelectric focusing and gradient slab acrylamide electrophoresis.

Blood was collected by insertion of an unheparinized capillary tube directly into the heart. The capillary tube was centrifuged at 5500 *g* for 15 min followed by serum removal. All samples were run fresh or stored at – 80°C before analysis. Specimens were not stored longer than 6 months.

Isoelectric focusing was performed on precast LKB slab acrylamide gels of pH ranges 3.5–9.5 (buffer system – anode 1 M H_3PO_4, cathode 1 M NaOH) and 5.5–8.5 (buffer system – anode 0.4 M HEPES, cathode 0.1 M NaOH). An LKB electrophoresis chamber and Pharmacia EPA 500/400 power supply were used for isoelectric focusing runs at 200 V (initial, 50 mA) to 300 V (final, 6 mA) for 3 h. Precast Pharmacia acrylamide gradient gels (4–30%) were used for serum and muscle protein electrophoresis. A Pharmacia GE-4 II chamber and EPS 500/400 power supply were used for electrophoresis at 75 V (initial, 75 mA) and 100 V (final, 80mA) for 18 h. The buffer system was 0.09 M Tris–0.08 M borate–0.003 M Na_2EDTA, pH 8.35.

General serum and muscle proteins were stained with 0.7% Buffalo Black NBR in 7% acetic acid for 3–4 h. Destaining was accomplished using successive changes of 7% acetic acid. Isozyme staining techniques are listed in Table 1.

Both parental species, the hybrid and an equal mixture of the parental species were used during each electrophoretic run. Saturated wicks were used for application of samples onto the isoelectric focusing gels. For gradient gels, 4 µl of serum and 20 µl of muscle homogenate were inserted into sample wells. Histochemical staining procedures followed those described in the literature [29–33] (Table 1).

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